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EFFECT OF ADRENOMEDULLIN ON PLACENTAL ARTERIES IN NORMAL AND PREECLAMPTIC PREGNANCIES AND THE MECHANISM OF ADRENOMEDULLIN-INDUCED RELAXATION

Sandra Mary Jerat ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Physiology

Edmonton, Alberta

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ABSTRACT

Adrenomedullin (ADM) maternal levels were determined, by radioimmunoassay, in normotensive and hypertensive pregnancies. The effect of ADM on normotensive and preeclamptic placental arteries, and the mechanism of ADM-induced relaxation were investigated using a wire myograph system. Plasma ADM levels were not different between normotensive pregnancy, pregnancy-induced hypertension, and preeclampsia. Nor was there any difference in ADM-induced relaxation in arteries from normotensive and preeclamptic pregnancies. ADM-induced relaxation was due both to nitric oxide release from the endothelium and to cAMP from the vascular smooth muscle cells. An unknown vasoconstrictive factor may be released from the endothelial cells. There is no compensatory increase in ADM levels in hypertensive pregnancies; this may contribute to the rise in blood pressure seen in hypertensive pregnancies. Retention of vasorelaxant activity in placental arteries from women with preeclampsia may attenuate increased placental vascular resistance seen in this condition.

To my family

and to the love of my life,

Peter Andrew

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TABLE OF CONTENTS

CHAPTER	PAGE
1. INTRODUCTION	1
Structure of Adrenomedullin	2
Regulation of Adrenomedullin production	3
Biological Effects of Adrenomedullin	5
Intracellular Mechanisms of ADM-Induced Vasorelaxation	8
Adrenomedullin Levels in Disease	12
Adrenomedullin Levels in Pregnancy	12
Normotensive Pregnancy	14
Preeclampsia	14
Nitric Oxide in Pregnancy	17
Nitric Oxide in Preeclampsia	17
Thromboxane A_2 in preeclampsia	20
The Placenta	20
Control of Blood Flow	24
Vasoactive Factors Implicated in Control of Placental Vascular Resistance	24
Proposed Experiments	33
2. METHODS AND MATERIALS	35
Experiment A – Maternal Plasma Levels of Adrenomedullin	36
Experiment B – Adrenomedullin-Induced Relaxation in Placental Arteries	38
Experiment C – Mechanism of Adrenomedullin-Induced Relaxation	41

3. RESULTS 46

Experiment A – Maternal Plasma Levels of Adrenomedullin 47

Experiment B – Adrenomedullin-Induced Relaxation in Placental Arteries 47

Experiment C – Mechanism of Adrenomedullin-Induced Relaxation 54

4. DISCUSSION AND CONCLUSIONS 65

Advantages and Disadvantages of the Wire Myograph Technique 74

Critical Review 77

BIBLIOGRAPHY 79

APPENDIX 108

LIST OF TABLES

		PAGE
CHAPTER 1		
Table 1	Multiple functions of adrenomedullin	6
CHAPTER 3		
Table 2	Characteristics of patient groups of experiment A	48
Table 3	Characteristics of patient groups of experiment B	50
Table 4	Characteristics of patient groups of experiment C	56
APPENDIX		
Table 5	Internal circumference: tension characteristics - computer program	112

LIST OF FIGURES

		PAGE
CHAPTER 1		
Figure 1	Nitric oxide biosynthesis and transcellular mechanisms	18
Figure 2	Anatomical arrangement of the placental vasculature	22
Figure 3	Schematic representation of blood flow through the intervillous space	23
CHAPTER 2		
Figure 4	Schematic representation of vascular branching the placental circulation	40
CHAPTER 3		
Figure 5	Maternal plasma adrenomedullin concentrations	49
Figure 6	U46619-induced contraction in placental arteries from normotensive and preeclamptic pregnancies (% constriction)	52
Figure 7	U46619-induced contraction in placental arteries from normotensive and preeclamptic pregnancies (mN/mm)	53
Figure 8	Effect of adrenomedullin on placental arteries from normotensive and preeclamptic pregnancies	55
Figure 9	Effect of nitric oxide inhibition on adrenomedullin-induced relaxation in normal placental arteries	57
Figure 10	Effect of endothelial removal on adrenomedullin-induced	58

relaxation in normal placental arteries

Figure 11 Effect of inducible nitric oxide inhibition on adrenomedullin-induced relaxation in endothelial denuded normal placental arteries 60

Figure 12 Effect of nitric oxide inhibition on adrenomedullin-induced relaxation in endothelial denuded normal placental arteries 61

Figure 13 Effect of nitric oxide inhibition and non-selective endothelin receptor antagonist on adrenomedullin-induced relaxation in endothelial intact normal placental arteries 62

Figure 14 Immunostaining for von Willebrand's Factor to confirm removal of the endothelium 64

CHAPTER 4

Figure 15 Hypothesized mechanisms of adrenomedullin-induced relaxation 69

APPENDIX

Figure 16 Internal circumference tension characteristics of a placental artery 113

LIST OF ABBREVIATIONS

1400W	N-(3-(Aminomethyl)benzyl) acetamidine - dihydrochloride
ADM	Adrenomedullin
ANF	Atrial natriuretic factor
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene-related peptide
CRLR	Calcitonin receptor-like receptor
D-NMMA	N^G-monomethyl-D-arginine
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
HEPES-PSS	4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered physiological salt solution
iNOS	Inducible nitric oxide synthase
L-NAME	N^{ω}-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
NO	Nitric oxide
NOLA	N^{ω}-nitro-L-arginine
NOS	Nitric oxide synthase
PATC	Phosphate alkaline treated casein
PIH	Pregnancy-induced hypertension
RAMPs	Receptor-activity-modifying proteins

RIA	Radioimmunoassay
SNAP	S-Nitroso-N-acetyl-D,L-penicillamine
SNP	Sodium nitroprusside
TxA_2	Thromboxane A_2
U46619	9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$
VSMC	Vascular smooth muscle cells

CHAPTER ONE

INTRODUCTION TO THESIS

AND

A BRIEF REVIEW

Adrenomedullin (ADM) was discovered in pheochromocytoma cells by its ability to elevate platelet cAMP (75), but has since been identified in other normal tissues. The characteristic actions of ADM are hypotension, natriuresis, and diuresis (66, 130). In the vasculature, ADM is secreted from both endothelial cells and vascular smooth muscle cells (VSMC) (61). There are two groups of receptors for ADM, both present on endothelial cells and VSMC. There are specific receptors for ADM, and non-specific receptors of calcitonin gene-related peptide (CGRP) that react with ADM (32, 101, 108). Depending upon the vascular bed and phenotype of the receptive cell, the mechanism of action of ADM may vary (147); ADM has been shown to exert its effects via stimulation of the nitric oxide (NO)-cGMP pathway (48, 160), activation of adenylate cyclase-cAMP (32), and through potassium channel activation (160). ADM is an important vasoactive peptide involved in cardiovascular function and control of body fluid. ADM levels rise during pregnancy (24, 27, 104). Pregnancy is characterized by a decreased mean arterial blood pressure despite an increase in blood volume and cardiac output (7, 37). ADM has been considered to play a role in the cardiovascular adaptation to pregnancy. Moreover, the increased levels seen in several hypertensive disorders, suggest a protective antihypertensive role of ADM (60, 69, 77, 122, 161).

Structure of ADM

Human ADM is 52 amino acid residues in length. It consists of a ring structure that is formed by an intramolecular disulphide linkage and a C-terminal amide structure. These structures are essential for its biological activity (76). ADM shares structural homology with CGRP, CGRP II, and amylin. They all have the property of this ring structure in

common (76). The human ADM gene is located on chromosome 11 and consists of 4 exons and 3 introns with a binding site for activator protein-2 and a cAMP regulated enhancer sequences (59).

Regulation of ADM Production

ADM is produced mainly from the endothelium and the VSMC (61). The secretion of ADM is under complex regulation and is influenced by various factors, either produced locally or circulating within the bloodstream as a hormone. The production of ADM from VSMC is augmented by several growth factors and cytokines, for example interleukin-1α and tumor necrosis factor-α (159), suggesting that ADM may have a role in vascular smooth muscle function in clinical conditions of septic shock, atherosclerosis, and inflammation (159). Indeed, plasma ADM levels rise 40-fold in endotoxemic patients (125). Several hormones also stimulate ADM production in VSMC; dexamethasone, hydrocortisone, aldosterone, and the thyroid hormones (103). ADM synthesis in endothelial cells is regulated by many of the same stimulants responsible for ADM production in VSMC. However, ADM production from endothelial cells is several times higher than that from VSMC (61).

Endothelin-1 (ET-1), a potent endothelium-derived vasoconstrictor peptide (85), also modulates ADM release. ET-1 can exert its effects via ET-A or ET-B receptors. ET-1 has been shown, through the ET-B receptor, to stimulate the release of ADM (64). Conversely, ADM has the ability to inhibit thrombin- and platelet derived growth factor-induced ET-1 production from VSMC, but not its basal release (78). This may reflect a

regulatory role of ADM in the modulation of ET-1 in various pathophysiological states (78).

Atrial natriuretic factor (ANF) is a hormone predominantly secreted from the atria of the heart in response to distention. It has many of the same functions as ADM, such as hypotension, diuresis, and natriuresis (82). ANF infusion, in healthy individuals, can stimulate the release of ADM causing natriuresis, diuresis, and a decrease in blood pressure (163). These two hormones may work in concert to lower blood pressure (163). Vesely et al. (1996) suggested that some of the responses to volume loading which were previously attributed to ANF may not be due directly to ANF, but may be secondary to the release of ADM (163). However, ADM significantly reduces stretch-induced secretion of ANF in an isolated atrium preparation (70). Stretch-induced ANF release is blunted during pregnancy and it has been postulated that ADM may be responsible (70), since ADM levels rise during pregnancy(24, 27, 104).

Hemodynamic perturbations such as volume overload (123, 140) and rapid ventricular pacing (65), have been shown to activate ADM gene transcription. In addition, physiological shear stress has been shown to be a stimulant of increased ADM mRNA expression in VSMC (13). More recently, ADM has been shown to increase in response to hypoxia; following exposure to a hypoxic environment, several organs such as the kidneys, lungs, hearts, brains, and liver, exhibit an increase in ADM mRNA and a subsequent increase in the ADM peptide itself (53).

The Biological Effects of ADM on Various Systems in the Body

ADM was discovered by its ability to increase rat platelet cAMP (75). Since its discovery, numerous biological functions of ADM have been discovered (Table 1) (33).

Cardiovascular System

In conscious sheep ADM has been shown to decrease blood pressure and significantly increase cardiac output and heart rate, with a marked decrease in total peripheral resistance (130). The hemodynamic effects of synthetic ADM administration in rats also reveal an increase in both heart rate and cardiac output (50). These cardiac actions of ADM may be due to a reduction in cardiac afterload due to the peripheral vasodilation, as well as a reflex sympathetic activation in response to the ensuing hypotension (50). A direct effect of ADM on the heart itself has also been suggested, considering this organ has a high level of mRNA expression for ADM (50), and the presence of mRNA for the putative ADM receptors in the heart (4).

ADM has structural homology with CGRP (75), a peptide which exhibits similar effects on the cardiovascular system. CGRP is a potent vasodilatory agent that lowers arterial blood pressure and has positive chronotropic effects on the heart (108). CGRP and ADM have been shown in rats to have very similar vasorelaxant activity on some vascular beds (75, 76).

Table 1. Multiple Functions of Adrenomedullin

Tissue or cell type	Bioactivity
Platelet	cAMP elevation
Vasculature	Vasodilation and hypotension
	Inhibition of endothelin secretion
	Inhibition of VSMC proliferation
	Stimulation of nitric oxide synthesis
Kidney	Diuresis and natriuresis
Lung	Pulmonary vasodilation
	Bronchodilation
Adrenal gland	Inhibition of aldosterone secretion
CNS	Inhibition of salt appetite
	Inhibition of water drinking
Pituitary	Inhibition of ACTH secretion
	Inhibition of vasopressin secretion
Pancreas	Inhibition of insulin secretion
Tumor cells	Proliferation of tumor cells

CNS, central nervous system; VSMC, vascular smooth muscle cell; ACTH, adrenocorticotropic hormone.

(From Eto T. *Clin Exp Pharmacol Physiol*. 26:371-380, 1999)

Kidney

ADM has been localized in the glomeruli, cortical distal tubules, and medullary collecting duct cells of normal canine kidneys (66). The renal excretory response to intrarenal infusion of ADM is a marked diuresis and natriuresis (66). These effects are a consequence of an increased glomerular filtration rate, fractional sodium excretion, and decreased distal tubular sodium reabsorption (66). Given these renal effects, ADM may have an important role in the regulation of sodium excretion.

Brain

The effect of ADM on the kidneys is complimented by the functions of ADM in the brain. Thus the increase in renal output of salt and water is accompanied by central inhibition of water intake (110) and salt appetite (148). However, the hypotensive effect of ADM administered into the peripheral circulation is not mimicked when ADM is administered directly into the brain (149). The effects of centrally administered ADM on blood pressure, heart rate, and renal sympathetic nerve activity are hypertension and tachycardia, and an initial decrease followed by a long-lasting increase in renal sympathetic nerve activity (145, 149).

Local or Paracrine Hormone

There is mounting evidence that ADM may act as a local or paracrine hormone. ADM mRNA has been found in significant concentrations in numerous tissues (124), but no definitive sites of ADM secretion from these tissues have been identified. This suggests that, under normal physiological conditions, ADM may act as a local or paracrine

hormone (124). Organs in which ADM mRNA is highly expressed are characterized by increased blood flow rates in response to infused ADM. Thus increased blood flow rates and ADM mRNA have been reported in the lungs, heart, spleen, kidneys, adrenal glands, and small intestine. By contrast, flow rates are unchanged in the brain, large intestine, and skin or decreased in skeletal muscle and in the testis (50); these organs have very low ADM mRNA. This supports the hypothesis that ADM acts as a local hormone (50) and that the vasodilatory actions of the ADM peptide may have a significant role in limiting resistance to blood flow in particular vascular beds. Since ADM secretion is high in the endothelium and the VSMC, and ADM receptors are present at these sites, it has been suggested that ADM acts directly on these cells via an autocrine or paracrine release (32, 61).

Intracellular Mechanisms of ADM-Induced Vasorelaxation

The vasorelaxation response to ADM has been reported in the resistance and capacitance vascular beds of various species. The effect of ADM infusion into the vasculature of the human forearm has been investigated (15, 118, 119), and has revealed potent vasodilatory actions of this peptide in humans. The vasoreactivity to ADM of isolated and perfused vessels has been investigated in the rat, cat, dog and pig, using such tissue as thoracic aortic rings, pulmonary artery rings, isolated cerebral arterioles and isolated coronary arteries (79, 106, 142, 165). Recently, the mechanisms of ADM-induced relaxation have begun to receive greater investigation. Thus far, the evidence suggests that the vasodilatory actions of ADM are initiated by three intracellular mechanisms: activation of the NO-cGMP pathway (48, 160), activation of adenylate cyclase-cAMP (32), and

potassium channel activation (160). Although all three mechanisms will be briefly discussed, the focus of attention in subsequent sections will be on the NO pathway. This is because it is this vasodilator pathway that has been heavily implicated in the control of resistance of vascular tone, plus it has received an exceptional amount of attention in the etiological theories of preeclampsia.

Signal transduction mechanisms following ADM receptor binding

Dose-dependent increases in cAMP stimulated by ADM have been identified in several different cells (21, 32). Consequently, cAMP is considered the major second messenger for ADM (75). The precise mechanism of ADM-induced relaxation in pig coronary arteries has been shown to be inhibition of intracellular calcium mobilization in the region of the cellular contractile apparatus, and a G-protein dependent decrease in calcium sensitivity of the contractile apparatus (80). G-proteins have been reported to mediate the ADM-induced generation of cAMP (80).

Although cAMP appears to be the major second messenger system for ADM-induced relaxation in several cell types, other signal transduction pathways for ADM are now recognized. Thus, ADM-induced relaxation has been shown to occur via activation of potassium channels (81, 142, 160). Potassium channels are also partially responsible for ADM-induced relaxation in the pulmonary vascular bed of the fetal sheep (160) and in the cerebral circulation (81). Although, further investigation is required to fully elucidate this mechanism of relaxation, Sabates et al. (1997) suggest a complex mechanism whereby ADM-induced relaxation is initiated by activation of adenosine receptors and

subsequent activation of potassium channels in the coronary vascular bed (142). However, the contribution to ADM-induced relaxation by activation of potassium channels may be in part due to NO activation, since NO can directly activate potassium channels in vascular smooth muscle cells (10).

NO has been shown to be an important mediator of ADM-induced relaxation; inhibition of NO reduces the vasorelaxation effects of ADM. The contribution of NO to ADM-induced vasodilation is dependent on the vascular bed and/or species being studied. In the pulmonary vascular bed of the rat, NO inhibition significantly inhibits ADM-induced relaxation whereas in the same bed of the cat there is no significant inhibition of ADM-induced relaxation (128). In the fetal sheep pulmonary artery, ADM-induced relaxation is almost completely abolished by NO synthesis inhibition (160). Furthermore, in the rat aorta and kidney, NO inhibition partially reduces ADM vasodilation (48). Production of NO from the endothelium is dependent on an increase in intracellular calcium within the endothelial cells (17). Indeed, ADM has been shown to increase the intracellular concentration of calcium in bovine aortic endothelial cells with a subsequent increase in NO (155). Therefore, on the basis of such evidence, NO appears to be an important mediator of ADM-induced relaxation despite species and vascular bed differences.

Receptors for ADM

Specific and non-specific (CGRP) ADM receptors are present on endothelial and VSMC (32). ADM stimulates cAMP formation in rat VSMC (32). The CGRP receptor antagonist (CGRP[8-37]) has inhibitory effects on cAMP formation (32), suggesting that ADM and

CGRP may interact with the same receptors (32). However, in vivo studies in the rat find that the CGRP receptor antagonist is unable to block the hypotensive effect of ADM (120), but is able to completely abolish the hypotensive effect of CGRP (120). This suggests that specific ADM-binding sites may be present within the cardiovascular system. cDNA has been isolated for the specific ADM receptor from rat lung tissue (68). This receptor may be the focus of non-CGRP effects of ADM within the circulation.

Recently it has been shown that the calcitonin receptor-like receptor (CRLR) has the ability to function as a CGRP or ADM receptor depending on the expression of receptor-activity-modifying proteins (RAMPs) 1 or 2. RAMPs are required to transport CRLR to the plasma membrane. RAMP 1 presents CRLR as a mature glycoprotein forming the CGRP receptor, whereas RAMP 2 glycosylates the CRLR and forms the ADM receptor. In cells where only one type of RAMP exists, there may be a pure receptor population (101). Kamitani et al. (67) confirmed the expression of the mRNA for RAMP2/CRLR in human endothelium and VSMC. Whereas, the expression of RAMP 1 was absent in the cells studied (67).

The Endothelium as an Important Site of Action for ADM

The endothelium is not only important for maintaining vessel wall integrity, but can influence vascular tone by releasing vasoactive agents, such as NO and ET (14). In rat aortic rings, canine mesenteric arteries, and canine femoral veins, removal of the endothelium attenuates ADM-induced vasorelaxation (6, 48). However, in canine femoral arteries ADM-induced relaxation is independent of the endothelium (6). It is apparent that

the importance of the endothelium in ADM-induced relaxation is dependent upon the vascular bed being studied.

ADM Levels in Disease

ADM is increased in several disease states such as, renal failure (58, 60), hypertension (60, 77, 161), heart failure (122), and primary aldosteronism (69). A relationship between ADM and norepinephrine has been suggested (60, 122) such that, in conditions where the sympathetic nervous system is enhanced, ADM would also be expected to increase. The actions of ANF and ADM have also been considered to be intimately related (60, 122). ANF has been shown to increase in congestive heart failure and renal failure (51). When body fluid volume increases ANF has been shown to increase. It has been suggested that there is a hypervolemic-induced increase in ADM (60). The increase of ADM that is found in these physiological and pathophysiological conditions may provide a protective mechanism to buffer the increase in blood pressure (60, 122).

ADM in Pregnancy: The Potential Significance of this Hormone

Normotensive pregnancy is generally characterized by a high blood volume, high blood flow, and low vascular resistance (37). During the course of pregnancy, blood volume and cardiac output increase by as much as 40-50% (7). Despite this, blood pressure decreases due to a decrease in total peripheral resistance. Increased circulating vasodilator factors may be responsible for the fall in total peripheral resistance. Since ADM is a potent hypotensive peptide, it has been considered as a potential candidate for the cardiovascular adaptation to pregnancy.

The concentration of ADM in maternal plasma increases during pregnancy in the human and in the rat (24, 27, 62, 104). Furthermore, fetoplacental tissues appear to be a significant site of synthesis of ADM during pregnancy (98). Immunoreactive ADM has been found in the placenta, predominately in the extravillous trophoblast cells, syncytiotrophoblast cells, and endothelial cells (98). ADM has also been found in amniotic fluid (92). The concentration of ADM in the umbilical vein and artery in normal pregnancies is not significantly different, suggesting that there is no placental clearance of ADM, or perhaps that the catabolism of ADM is made up by an equal amount of production (52).

The role of ADM in preeclampsia is complex. Maternal plasma levels of ADM in preeclamptic women compared to uncomplicated pregnancies have been reported to be either increased (83), decreased (47), or the same (26, 104). ADM levels in amniotic fluid and umbilical vein plasma are reported to be significantly increased in preeclampsia (26). Immunoreactive ADM was found to be increased in fetal membranes and in the umbilical artery of patients with pregnancy-induced hypertension (PIH) (96). ADM has also been shown to attenuate the hypertension induced by a nitric oxide synthase (NOS) inhibitor during late pregnancy in an animal model of preeclampsia (97). On the basis of this evidence, ADM may play a protective role in preeclampsia. To further examine the role of ADM in pregnancy, an understanding of the physiology of normotensive pregnancy and the pathophysiology of preeclampsia is necessary.

Normotensive Pregnancy

As mentioned previously, normotensive pregnancy is generally characterized hemodynamically as a high blood volume, high blood flow, and low vascular resistance state (37). Vasodilator substances, which have been implicated in causing the decrease in total peripheral resistance, include prostacyclin, prostaglandin E, and NO. These vasodilators may also have a role in making the vasculature less sensitive or hyporesponsive to vasoconstrictor agents. Indeed, in a study by Gant et al. (1973) normal pregnancy was reported to exhibit resistance to the pressor actions of angiotensin II, but in PIH this pressor resistance was absent (38). Moreover, the loss of this refractory response to angiotensin II could be demonstrated before the onset of hypertension (38). It has also been suggested that a deficiency in vasodilator peptides may be an additional contributing factor to the pathophysiology of preeclampsia (37).

Preeclampsia

Preeclampsia is a multi-system disorder that is present in approximately 6% to 8% of all human pregnancies (12). Preeclampsia is a state of low blood volume, high blood pressure, and high vascular resistance compared to normotensive pregnancy (37). The hallmark characteristics of preeclampsia are persistent hypertension, proteinuria, and edema (37). As expressed earlier, the adaptation of the cardiovascular system observed in normal pregnancy, appears to be deficient in preeclampsia (37).

The hypertension seen in preeclampsia, although a major characteristic of the disease, is not the pathogenic factor that causes the multi-system syndrome, but is a manifestation of

the disease. The pathophysiology of preeclampsia is not fully known. The common pathological feature of the disease is endothelial cell dysfunction. The cause of this alteration in endothelial cell function is unknown.

Endothelial Cell Dysfunction

Evidence to support endothelial cell dysfunction in preeclampsia is mounting. Endothelium-dependent vasodilator responses in resistance arteries dissected from subcutaneous fat tissue of women with preeclampsia or normotensive pregnancies have been compared. There is an attenuated relaxation response in arteries from preeclamptic women (100). VSMC respond equally to the endothelium-independent vasodilator, sodium nitroprusside (SNP), in both normotensive and preeclamptic pregnancies suggesting that the sensitivity of VSMC to NO is not impaired in preeclampsia (100). Further complimentary evidence has been gathered from other vascular beds within the placenta. For example, an attenuated relaxation response to bradykinin, a vasoactive agent which relaxes VSMC by an endothelium-dependent mechanism, has been reported in myometrial arteries from preeclamptic pregnancies (2). In addition, when myometrial resistance arteries from normotensive pregnancies were exposed to plasma from woman with preeclampsia, the arteries exhibited an attenuated response to bradykinin-induced relaxation (3, 49). This is supportive of the theory that there is a plasma-borne factor present in preeclampsia that causes endothelial cell dysfunction (3, 49). Altogether, this evidence suggests that endothelium-dependent mechanisms may be dysfunctional in preeclampsia.

The factor or factors, present in plasma from women with preeclampsia, that are responsible for the dysfunctional endothelium-dependent vasoreactivity, is unknown at this point. The origin of this factor has been suggested to be the placenta itself. The placenta is unquestionably involved in the pathogenesis of preeclampsia considering that delivery of the placenta in preeclampsia eradicates all symptoms (136). It is well known in preeclampsia that there is poor utero-placental perfusion (138). During the development of the placenta there are normal morphological changes that must occur to alter the vessels at the fetomaternal interface, in order to accommodate a high rate of blood flow to supply the growing fetus. The main morphological change in the microcirculation is the invasion of the maternal spiral arteries with the migrating trophoblast cells. These trophoblast cells replace the endothelium of the spiral arteries and the medial layer, resulting in destruction of maternal neural control of these arteries (23). Consequently, the spiral arteries lose their neural-derived vascular reactivity. By contrast, in preeclampsia, complete invasion of the spiral arteries is absent, leaving intact neuronal control (102, 131). Therefore, these arteries remain muscular, non-dilated, and capable of contraction. Failure of trophoblast invasion results in high tension within these resistance arteries that leads to decreased blood flow to the placenta (23). As a consequence of poor blood flow there is a predisposition to placental ischemia. It is possible that placental ischemia may be the initiating factor that causes production of the unknown damaging factor(s) that alters endothelial cells. Recently, Page et al. (2000) suggest that, in preeclampsia, neurokinin B is released in excess from the placenta and could potentially be a contributing factor to the pathogenesis of preeclampsia (129).

Neurokinin B causes contraction of the portal vein and of the mesenteric bed (129). In vivo experiments in rats demonstrate an increase in mean arterial blood pressure (129).

Nitric Oxide in Pregnancy

NO has commanded much attention in studies of both normal and abnormal pregnancy. NO biosynthesis increases in normal pregnancy (Figure 1) (146), as demonstrated by the increase in urinary excretion and plasma concentration of the stable NO metabolite, nitrate, and increased urinary excretion of 3',5'-cyclic monophosphate (cGMP, second messenger of NO) (19, 146). The importance of this increase in NO has been illustrated in rats by the effects of blockade of NO synthesis by L-nitro-arginine methylester (L-NAME). Inhibition of NO synthesis produces hypertension, proteinuria, thrombocytopenia, and intrauterine growth retardation, all of which are characteristic of preeclampsia (105).

Nitric Oxide in Preeclampsia

The shear stress associated with blood flow through vessels, is a major stimulus for NO production by the vascular endothelium. It has been shown that flow-mediated NO release is enhanced in normal pregnancy compared to non-pregnant controls (16, 30). However, in preeclampsia, flow-mediated vasorelaxation is reduced compared to normal pregnancy (16). Hence, an abnormality in the flow-mediated NO pathway of vasorelaxation may be a cause for the cardiovascular dysregulation that arises in preeclampsia.

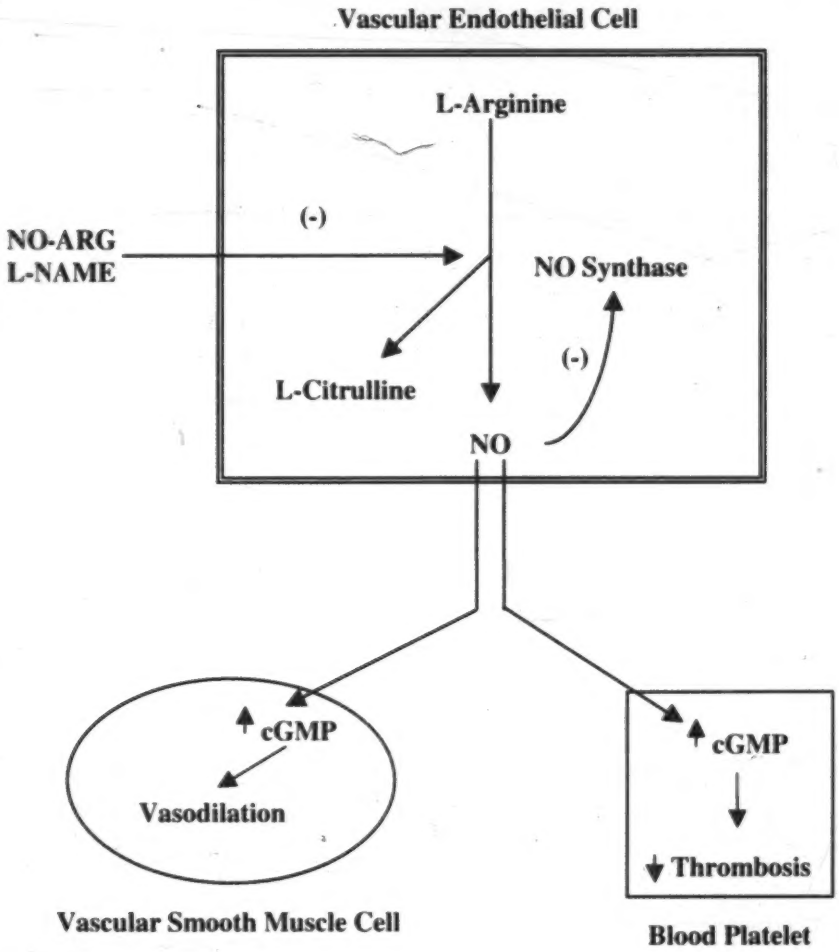

Figure 1. NO biosynthesis and transcellular mechanisms of NO communication with its target cells (from Salas S.P. *Biol Res.* 243:250-250, 1998).

Although, derangement of the NO-system has thus been implicated in preeclampsia (12), nitrate concentrations in amniotic fluid are higher in preeclamptic pregnancies compared to normotensive pregnancies (25). Levels of nitrate in the amniotic fluid may be a reflection of fetal urinary excretion and production from both the placental and fetal membranes, so the relevance of this finding may not be obvious (25). A reduction in urinary excretion of NO metabolites in women with preeclampsia compared to normotensive pregnancies has been reported suggesting a decrease in NO production in women with preeclampsia (22). There is also enhanced endothelial nitric oxide synthase (eNOS) expression in endothelial cells from stem villous vessels from placentas obtained from preeclamptic pregnancies (25). Since, increased expression of eNOS is not an indication of actual enzymatic activity, these results should also be evaluated with caution. Moreover, elevation in the vasculature may suggest a compensatory mechanism to counterbalance the release of excessive quantities of vasoconstrictor agents in preeclampsia, such as ET-1 and thromboxane A_2 (TxA_2) (25).

Endothelial cells exposed to plasma from women with preeclampsia show an increase in NO production and NOS activity compared to plasma from normotensive pregnancies (5). Although such an increase might appear at first sight to be beneficial in preeclampsia, NO could actually damage the endothelium and alter its function through its reaction with free radicals and subsequent formation of peroxynitrite (5, 117, 139).

Thromboxane A_2 in Preeclampsia

Preeclampsia has been proposed to be associated with a shift in the balance between vasoconstrictor and vasodilator eicosanoids in favor of the former (89). Elevated TxA_2 levels may derive from the placenta in preeclampsia (164). There is also evidence for altered vasoreactivity to eicosanoids in the resistance vasculature of preeclamptic placentas (135). In platelets from women with preeclampsia, receptors for TxA_2 have been found to be either increased or the same in comparison to normotensive pregnancies. However, both studies show an increased sensitivity to TxA_2 in platelets obtained from women with preeclampsia compared to normotensive pregnancy (28, 88). In a placental lobule perfusion preparation the increase in pressure created by 9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$ (U46619), a TxA_2 mimetic, is greater in placentas from normotensive pregnancies than from women with preeclampsia (135). These results suggest that placentas from preeclamptic pregnancies are less sensitive to the vasoconstrictor effects of TxA_2. This could be a protective mechanism to counter increased levels of TxA_2 in preeclampsia, or could reflect a downregulation of receptors for TxA_2 (135).

The Placenta

The Role of the Placenta

The placenta is a critical organ for proper growth and development of the fetus. Its main functions are to provide nutrient, waste, and gaseous exchange for the developing fetus. This organ also plays a role in providing secretory and regulatory functions necessary for the maintenance of pregnancy. The importance of the placenta and its viability is echoed

by the fact that, when placental blood flow is inadequate (for example due to increased fetoplacental resistance), a poor pregnancy outcome is typical.

Fetoplacental Circulation and Structure

In the human, the fetoplacental circulation is made up of two umbilical arteries, one umbilical vein, chorionic plate arteries and veins, and fetal stem villous arteries and veins (134). The umbilical vessels branch, up to 4 to 5 times, and give rise to the chorionic plate vessels (72). The chorionic plate vessels then branch to form the stem villous vessels that continue to divide to form the placental villous vascular tree. Within the human placental villous tree there are three types of villi: stem villi, terminal villi, and immature villi. The stem villi are responsible for mechanical stability of the tree, the terminal villi are the site of gaseous and nutrient exchange, and the function of the immature villi remain to be fully elucidated (71). Each main stem villous supplies blood to one cotyledon, of which there are approximately sixty present in one placenta (133). Figure 2 shows the arrangement of the placental circulation in a single cotyledon, showing both maternal and fetal sides of the circulation (153). Figure 3 indicates the direction of maternal blood flow within the intervillous space (133).

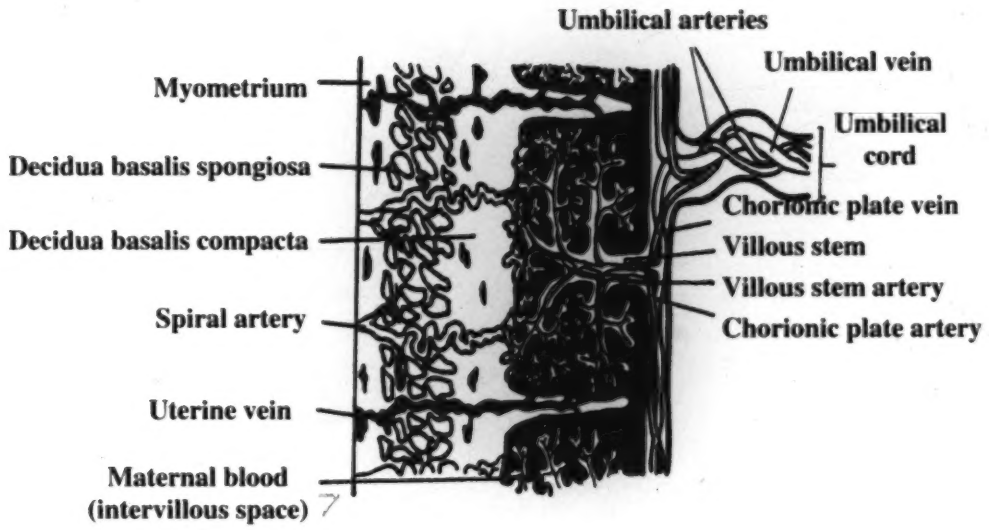

Figure 2. Schematic representation of the anatomical arrangement of the vasculature in a single lobe of the human placenta. This figure demonstrates the relationship between maternal and fetal circulations within the placenta (from Sastry R.B.V. *Troph Res.* 2:289-304, 1987).

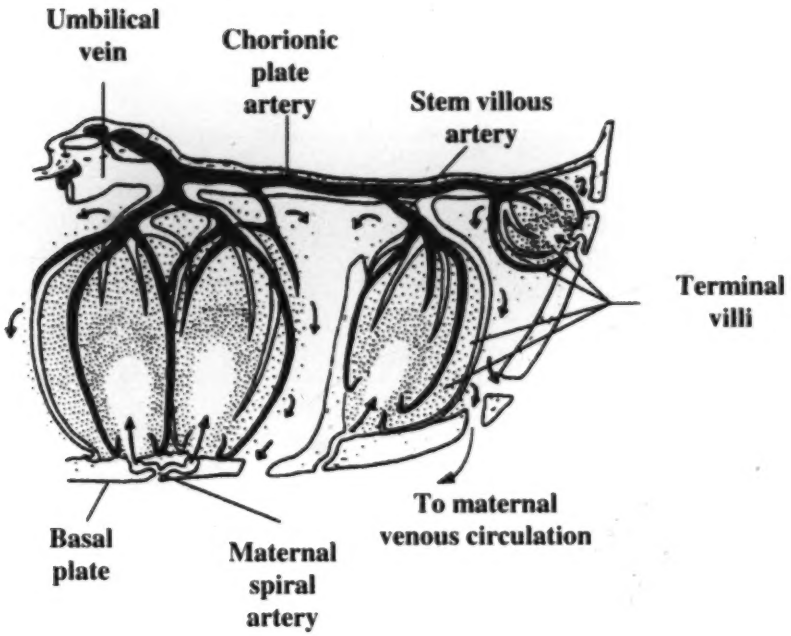

Figure 3. Schematic representation of the direction of circulation within the intervillous space (from Poston L. *Experimental Physiol.* 82:377-387, 1997).

Control of Blood Flow

The placental circulation is characterized by low vascular resistance and high rate of blood flow. The human placenta receives approximately 20% of fetal biventricular cardiac output at term (133). The determinants of blood flow in the placenta are equivalent to those in other vascular beds, and includes the number, length, and caliber of arteries, and the viscosity of blood. Poiseuille's Law describes this relationship:

$$R=8\eta l/\pi r^4$$

where R is resistance, r is radius, η is blood viscosity, and l is vessel length (133). The umbilical arteries are large in diameter and are not major contributors to placental vascular resistance. However, the stem villous arteries branch extensively to ultimately form the terminal villi for gas exchange. The main stem villous arteries and their branches contain smooth muscle and therefore are most likely responsible for resistance in the normal placenta (71, 111, 134).

A unique feature of the normal human placenta is that it is devoid of any autonomic innervation. Therefore, neuronal control plays no role in the regulation of vascular resistance within the placental circulation (36, 137). Hence, local vasoactive factors are the primary determinants of vascular resistance within the placenta (133, 134).

Vasoactive Factors Implicated in the Control of Placental Vascular Resistance

Oxygen

Many studies examining vascular function in placental arteries have been performed in 95% oxygen (41, 55, 93-95, 143, 144). This creates partial pressures of oxygen (PO_2)

greater than 300 mmHg. The oxygen tension within the placenta in late gestation is only about 28.0 mmHg (121). Many investigators have ignored the importance of this fact. McCarthy et al. (1994) found that gassing physiologic salt solution with 5% CO_2 in nitrogen caused an approximate physiologic oxygen tension of 38.03 ± 7.50 mmHg (99). Such an experimental preparation would appear to more closely meet the goal of reproducing the physiological conditions within the placenta compared with studies that have been performed in 95% oxygen. Changes in PO_2 may influence placental blood flow. Therefore, putting aside the actual validity of reproducing low oxygen tension in placental experimental preparations, the effect of altered oxygen tension on vascular function has been investigated as a possible regulator of placental vascular resistance. In an isolated umbilical artery preparation, an increase in PO_2 from 16 mmHg to 120 mmHg created a transient contraction mediated by TxA_2 (162). In addition, there was increased sensitivity to 5-hydroxytryptamine and 5-carboxamidotryptamine in high O_2 which was also mediated by TxA_2 (162). Hence, the vascular responsiveness to vasoactive agents is capable of being affected by the oxygen tension within these placental tissues. On the other hand, increased oxygen tension does not alter the vasoconstrictor effect of ET-1 on the umbilical artery (91). Therefore, the altered vasoreactivity to changes in oxygen tension may only be in regard to some vasoactive agents. In addition, oxygen-induced contraction to vasoactive agents appears to be predominantly important in the umbilical vasculature, since no effect of oxygen tension has been reported in the villous vasculature. Studies in villous arteries show no difference to U46619-induced contraction in low or high oxygen tension conditions (99). However, there is a decrease in sensitivity to vasorelaxation induced by SNP in high oxygen conditions, despite there being no

difference in the maximum relaxation response (99). Hence, oxygen appears to have an effect on vascular function within the placenta at supraphysiologic oxygen tensions. On this basis, oxygen tension should be taken into consideration when studying the effects of various vasoactive agents on the placental vasculature.

Vasoconstrictors

The most potent endogenous vasoconstrictor in the placenta is ET-1 (99). At low concentrations, ET-1 stimulates the release of endothelium-dependent vasodilators. It has been suggested that, at this low concentration, ET-1 may actually contribute to decreasing the vascular resistance within the placenta (144). However, at high concentrations, ET-1 reveals its potent vasoconstrictor activity (144). Other vasoconstrictor agents such as angiotensin II, prostaglandin E_2, and prostaglandin $F_{2\alpha}$ also cause vasoconstriction within the placenta, but are less potent than ET-1 (99). ET-1 is a vasoconstrictor throughout the fetal-placental vascular tree and aids in the maintenance of placental vascular resistance (91). Fetal-placental vessels possess TxA_2 receptors and therefore TxA_2 has been investigated for its potential role in determining the vascular tone of placental vessels (54). U46619, a TxA_2 mimetic, has been shown to be a potent vasoconstrictor of the placental circulation (144). It does appear that the vasoconstrictor effects of ET-1, angiotensin II, and 5-hydroxytryptamine are mediated, at least in part, by TxA_2 (54). However, these experiments were carried out in conditions of high oxygen tension (95% oxygen and 5% carbon dioxide) which may have altered the vascular response to TxA_2, so the conclusions of this study should be viewed with caution. Nevertheless, this study highlights the complexity of mechanisms that control vascular tone within the placental

circulation, where many vasoactive agents may act individually or in concert through common constrictor/dilator agents to cause a particular vascular response.

Vasodilators

The Role of NO in the Placental Circulation in Normotensive Pregnancy

Both the calcium-dependent (constitutive NO synthase) and calcium-independent (inducible NO synthase (iNOS)) isoforms of NOS are present in placental villi (39, 156, 157), the constitutive form of NOS being more prevalent (114). It is reasonable to assume that the role of iNOS in the placenta is to produce NO for immune defense. Positive immunostaining for iNOS has been reported in the endothelium of terminal villous and stem villous vessels of placentas from both normotensive and preeclamptic pregnancies (115, 116); there was no apparent difference in intensity of immunostaining between the two conditions (116). The umbilical cord, chorionic plate, and stem villous vessels all demonstrated positive immunostaining for eNOS (42, 113). Thus, the machinery necessary for production of NO is present throughout the conduit and resistance vasculature of the placenta.

The fetoplacental circulation has the ability to generate NO, the basal release of which may maintain the low vascular resistance characteristic of the placental circulation (112). Infusion of methylene blue (a soluble guanylate cyclase inhibitor) has been reported to increase vascular resistance in a placental lobule perfusion model (112). Moreover, NO inhibitors, N^{ω}-nitro-L-arginine (NOLA), hemoglobin (NO inactivator), and methylene blue increase basal perfusion pressure and increase U46619-induced contraction within

this vascular preparation (73). Treatment with N^G-nitro-L-arginine methyl ester (L-NAME) also increases basal tension in isolated vessel preparations (99). On the basis of this evidence alone, NO appears to have a primary role in the maintenance of low vascular resistance, as well as the attenuation of constrictor responses within the placenta (73). Flow-mediated release of NO appears to be an important feature in maintaining the low-resistance of the placental circulation (84). In contrast, inhibition of eicosanoid synthesis (with particular inhibition of prostacyclin), has been shown to have no significant effect on basal perfusion pressure or potentiation of the vasoconstrictor effect of U46619 (73, 99). This suggests that there is no basal release of dilator or constrictor prostanoids in the placenta and moreover, signifies the importance of NO in determining the basal tone within the placental circulation.

The Significance of NO in the Placental Circulation in Preeclamptic Pregnancies

Considering NO is important in maintaining the low vascular resistance that is characteristic of the placenta, it has been suggested that a deficiency of NO could be an important pathological feature of pregnancies complicated with preeclampsia. Thus reduced eNOS in the umbilical artery in complicated pregnancies (141), and reduced NOS activity, assayed in homogenized tissue, in the villous tissue from preeclamptic pregnancies (107) may contribute to the increased resistance seen in preeclamptic pregnancies and support the proposal that endothelial dysfunction is the characteristic underlying cause of preeclampsia. On the other hand evidence against this proposal exists in a study that reports *elevated* levels of NOS in whole-placenta tissue extracts from pregnancies complicated with preeclampsia (141). An argument may be made that

elevation in NOS does not necessarily go hand-in-hand with increased NO bioavailability. Taken altogether, perhaps these studies most importantly highlight the value of investigating the appropriate resistance vessels when proposing the significance of a particular observation within the placental vascular bed. In preeclampsia, where there is an increase in placental vascular resistance (74), it is the stem villous arteries that determine resistance within the placental circulation; the umbilical vessels are conduit vessels that are not involved in determining resistance within the placenta (133). Therefore, studies that use non-resistance vasculature (such as the umbilical artery) or whole tissue extracts to examine potential differences in preeclampsia versus normotensive pregnancy have to be evaluated with caution.

Increased NO: possible benefits and consequences - Increased eNOS gene expression is seen in the fetoplacental vasculature of women with preeclampsia compared with normotensive pregnancies (115). Although gene expression may not always directly correlate with the quantity of the protein or the activity of the final enzyme itself, these data may reflect an underlying feature, namely that the increased gene expression of NOS may be an adaptive response to the increase in vascular resistance and poor tissue perfusion that occurs in preeclampsia. Curiously, the reported elevation in eNOS gene expression in preeclamptic fetoplacental vasculature is at odds with the findings of reduced eNOS reported in the umbilical artery (141) and villous tissue from preeclamptic pregnancies (107). In placentas of preeclamptic pregnancies, the terminal vessels of the villous vascular tree stain positive for eNOS staining in the endothelium whereas, no staining for eNOS has been found in normal placentas (42). Furthermore, staining in the

syncytiotrophoblast layer is more diffuse and superficial in preeclamptic placentas compared to the punctuate staining in normal placentas, possibly due to vascular alterations or damage attributable to this condition (42). Other studies report no difference in NOS activity in the syncytiotrophoblast layer between these groups (18). Nevertheless, NO production in the uteroplacental, fetoplacental, and peripheral circulations is reported to be higher in preeclampsia compared to normotensive pregnancies (127). Again, the enhanced production of an important vasodilator agent may be a compensatory mechanism to offset the pathophysiology of preeclampsia (127). It should not be forgotten that increased NO can be detrimental. NO can react with superoxide to form the peroxynitrite anion. Peroxynitrite is an oxidant that has cytotoxic effects on the endothelial cells. Nitrotyrosine residues, which are indicators of peroxynitrite production, have been found to be increased in the placentas from women with preeclampsia (117). The formation of peroxynitrite leads to the scavenging of NO and thus the removal of any beneficial effects of NO. Therefore, it is possible that in preeclampsia, NO synthesis may be increased but its degradation is hastened by peroxynitrite formation. Hence the bioavailabilty of NO in the placental circulation in preeclampsia may be decreased, not due to decreased synthesis but because of the formation of peroxynitrite.

iNOS - Conrad and Davis (1995) also found that iNOS expression was considerably less than that of eNOS in many placental tissues. Increased iNOS expression would be expected in preeclamptic tissue samples due to the elevation in cytokine mediators (a major stimulus for iNOS activation) in preeclampsia (18). Thus the lack of a dramatic

elevation in iNOS raises further questions about the placental vasculature in preeclampsia. The importance of this finding may be tempered given that the inducible form of NOS may only be a minor component of NOS expression in the placenta, since it has only been found in small amounts in comparison to eNOS (18).

NO Sensitivity - Endothelial cell injury is viewed as a possible pathogenic mechanism that occurs in preeclampsia. As a consequence of this cellular damage the vessels may have an altered production and response to vasoactive agents within the placental circulation. For example, women with preeclampsia have an increased sensitivity to pressor agents (38). There is evidence for dysfunctional vasodilator mechanisms too. No difference in the vasorelaxant response to SNP has been observed in chorionic veins from preeclamptic placentas. This suggests that the vascular changes in preeclampsia do not affect VSMC function (9). In contrast however, another NO donor, S-Nitroso-N-acetyl-D,L-penicillamine (SNAP), does produce an attenuated relaxation response in chorionic arteries and veins from preeclamptic pregnancies compared to normotensive pregnancies (44). The dissimilar vascular response to SNP and SNAP reported in these studies may be due to differences in the respective properties of each vasoactive agent. Despite SNP and SNAP both being endothelium-independent NO donors, the vasoactive effect of each agent may be strongly influenced by their individual biochemical characteristics as NO donors (126). It has been reported that SNP and SNAP donate different NO redox species (35, 158), and that guanylate cyclase activation (the primary intracellular pathway for NO-induced vasorelaxation) has a preference for a particular redox form (29). In

conclusion, there is thus evidence for a difference in vasoreactivity to NO between normal and preeclamptic placental resistance vessels.

Importance of the Endothelium in the Placental Circulation

The endothelium is a significant source of vasoactive agents (99, 143, 144). Despite the fact that there is no external neuronal control of the placenta (36, 137), acetylcholine induces relaxation in small placental arteries (143). Acetylcholine binds to muscarinic receptors, located on the endothelial cells, and causes the release of NO (151). Thus acetylcholine-induced relaxation requires the presence of the endothelium. In small placental vessels, removal of the endothelium abolishes relaxation induced by classic endothelium-dependent vasodilators such as acetylcholine, adenosine diphosphate, and histamine (143). It should be noted that not all investigators have found a relaxation response to acetylcholine in small placental arteries (99). The reasons for this are unknown.

Endothelial cells can also modulate the vasoactive response to vasoconstrictors. For example, endothelial-denuded ring preparations of placental arteries produced greater ET-1-induced vasoconstriction compared to the endothelium-intact arteries (144). The actions of the vasoconstrictor, ET-1, appear to be modulated by the presence of the endothelium. Consequently, it may be proposed that, in response to ET-1, the endothelial cells release a vasodilator substance, possibly NO, which blunts the vasoconstriction. Endothelium-dependent relaxation occurs in response to low doses of ET-1, and this has been proposed to be partially mediated by NO and an ATP-sensitive K^+ channel activator (144). In

summary, these studies illustrate the importance of the endothelium in determining the vascular tone that is generated within the placental resistance vessels, and demonstrate how many vasoactive agents mediate their effects on vascular tone through interaction with the endothelium.

Proposed Experiments

Since the placenta is devoid of autonomic innervation (137) locally produced vasoactive factors are essential in maintaining the low vascular resistance normally present within the placental circulation. A paracrine or autocrine mode of action of ADM has been inferred from the elevated concentrations found in fetoplacental tissues (98). We hypothesized that maternal plasma ADM levels should, as in other hypertensive disorders (60, 69, 77, 122, 161), be higher in hypertensive pregnancies than in normotensive pregnancies, and that the higher placental resistance found in preeclamptic pregnancies results, not from a lack of locally produced ADM, but from blunted activity of ADM on placental resistance vessels. Therefore we sought to evaluate maternal plasma ADM levels and the effect of ADM on placental arteries from both preeclamptic and normal pregnancies.

Our second objective was to elucidate the mechanism of ADM-induced relaxation in placental arteries from normotensive pregnancies. We hypothesized that inhibition of NO would attenuate ADM-induced relaxation in endothelium-intact arteries and that endothelial denudation would significantly reduce ADM-induced relaxation. Preliminary studies suggested that ADM-induced relaxation was partially dependent on NO, but that

the NO release was not derived from the endothelium. Consequently, we considered non-endothelial sources of NO ie. NO production from VSMC by way of iNOS and/or eNOS. Thus, we hypothesized that iNOS and/or eNOS inhibition in endothelial denuded arteries would attenuate ADM-induced relaxation. Follow-up preliminary data suggested that the latter two sources of NO were not significant mechanisms in ADM-induced relaxation. As a result, we considered that, upon ADM stimulation, the endothelium might be releasing a vasoconstrictive factor such as endothelin (ET). We hypothesized that an endothelin receptor antagonist plus NO inhibition would cause greater ADM-induced relaxation than NO blockade alone, and that endothelin receptor antagonism alone would exaggerate the relaxation response. The focus of this part of the study was thus to elucidate the mechanism of ADM-induced relaxation in normal placental arteries focusing on the role of the endothelium, nitric oxide, and opposing vasoconstrictive factors released by the endothelium.

CHAPTER TWO

MATERIALS AND METHODS

Experiment A - Maternal Plasma Levels of ADM

Patient Population

This study was approved by the Institutional Ethics Review Board. Women with preeclampsia were defined using the criteria of hypertension and proteinuria. Hypertension was defined as >140/90mmHg on 2 occasions at least 6 hours apart and occurring after the 20th week of gestation. Proteinuria was defined as ≥1+ by the dipstick method. The women with pregnancy-induced hypertension (PIH) were defined using the above criteria for hypertension and no proteinuria. The women with uncomplicated pregnancies were normotensive (<140/90mmHg) and presented with no proteinuria. No patient was known to have a history of chronic hypertension, liver, renal, or metabolic disease.

Sample Collection

After informed consent was obtained, blood samples were collected on ice into tubes containing EDTA plus 500KIU aprotinin per 5mL tube. Women had a blood sample drawn on one occasion within one of the three time intervals. Samples were centrifuged within 30 minutes of collection. Plasma was separated and stored at -70°C until ready for extraction.

Plasma Extraction

Plasma samples (1mL) were extracted using the method described by Lewis et al (86). Briefly, plasma was mixed with an equal volume of phoshphate alkaline-treated casein (PATC) buffer. Sep-Pak C-18 columns (Waters Corporation, Milford, MA., USA) were

pre-equilibrated with 5mL of methanol followed by 10mL of 0.9% saline. The plasma-buffer mixture was added and the columns were washed with 5mL of 0.9% saline. ADM was eluted with 2mL 80% isopropanol/0.013mol/L HCl into a tube containing 10uL of 1% triton X-100. The eluate was dried under nitrogen and the extract stored at -70°C until ready for radioimmunoassay. Extraction efficiency was measured by the addition of a known amount of unlabelled ADM to plasma with a known amount of endogenous ADM. Recovery was calculated by comparing measured ADM levels with a control of RIA buffer with the same amount of ADM added without extraction. Recoveries of unlabelled ADM in this extraction procedure were 85% in normotensive pregnant plasma, 81% in preeclamptic plasma, and 72% in non-pregnant plasma. There was no significant difference in recoveries between these groups. Extraction efficiency was measured in non-pregnant women to ensure the state of pregnancy did not alter the extraction efficiency.

Radioimmunoassay (RIA)

Prior to analysis, plasma extracts were reconstituted with 250uL of RIA buffer. RIA materials and methods were supplied by Phoenix Pharmaceuticals (Mountain View, CA). Each sample was assayed in duplicate for human ADM. Plasma samples from all three groups were analyzed in the same assays. The intra- and inter-assay coefficients of variance were 7.5% and 10.0%, respectively. Data were not corrected for peptide recovery. Information provided by Phoenix Pharmaceuticals, gave details regarding assay sensitivity and specificity. Sensitivity of the assay was an IC_{50} value of 10pg/tube. The primary antibody provided in this assay was specific for human adrenomedullin. Other

vasoactive compounds (ADM 13-52 (human), CGRP (human), and CGRP II (human)) did not cross-react with the antibody.

Solutions

The PATC contained 0.05mol/L phosphate buffer pH 7.4, 0.1% alkali-treated casein (ATC), 0.1% triton X-100, 0.1% sodium-EDTA, 0.2% sodium azide. ATC was prepared following a method previously described (90). The RIA buffer contained 19mmol/L sodium phosphate, 81mmol/L dibasic sodium phosphate, 0.05 mol/L sodium chloride, 0.1% bovine serum albumin, and 0.01% sodium azide.

Statistical Analysis

Data were analyzed using the Mann-Whitney Rank Sum Test. Significance was accepted at $P<0.05$.

Experiment B - ADM-Induced Relaxation in Placental Arteries

Tissue Preparation

Placentas were obtained after vaginal delivery or caesarian section from both normotensive and preeclamptic pregnancies. It has been shown that the mode of delivery has no effect on the responsiveness of placental vessels (41). Immediately after removal of the placenta, a piece was cut from a macroscopically normal cotyledon and placed in cold 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered physiological salt solution (HEPES-PSS). Small stem villous arteries, averaging 300 μm in diameter and 2 mm in length, were then dissected from the placental specimens under a light microscope

to remove surrounding trophoblast and connective tissue. Figure 4 shows the arrangement of the placental circulation, specifically showing the placement of the stem villous arteries that were isolated in this study (152). The stem villous artery was chosen because it is the major site of vascular resistance in the placenta; the umbilical and chorionic plate vessels do not contribute greatly to vascular resistance (133). The isolated arteries were cut into rings and mounted on a wire myograph system (109). Two 25 μm tungsten wires were passed through the lumen of the artery to secure the vessel to the supports in a 5 mL organ bath. One support was connected to a micrometer and the other to the isometric force transducer. The arteries were bathed in HEPES-PSS at 37°C and at pH 7.4 for 30 minutes prior to any manipulation and throughout the duration of the experiment. The passive tension and internal circumference characteristics of the artery were then determined (109). Briefly, the diameter of the artery was progressively increased in stepwise increments while the force generated was recorded. The Laplace relationship was used to estimate transmural pressure. The stem villous arteries were then set to 90% of the internal circumference they would have had when relaxed under a normal physiological transmural pressure of approximately 40 mmHg. Arteries were set to 90% because this value was previously found to produce the optimal active tension (see appendix for procedure). Arteries were then allowed to equilibrate for 30 minutes. At the end of each experiment the viability of the arteries was tested using a potassium-chloride depolarizing solution.

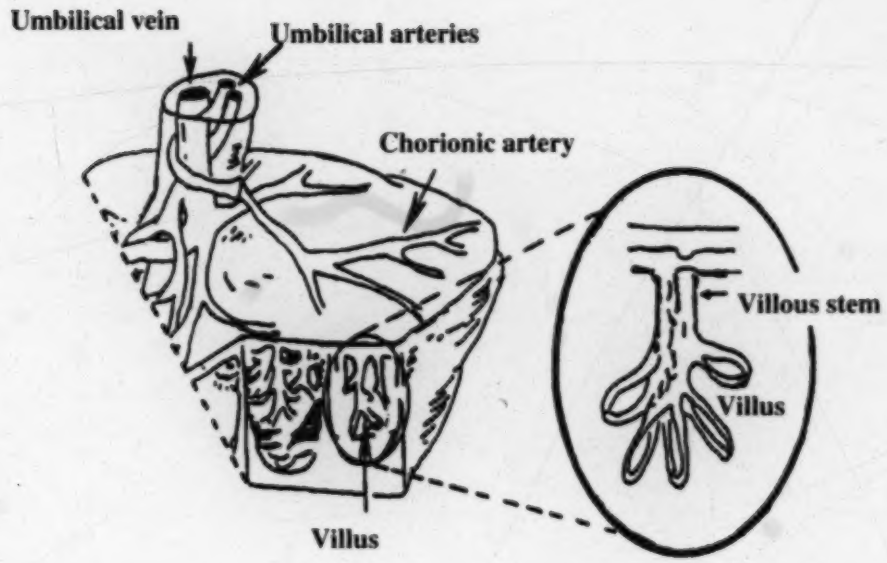

Figure 4. A schematic figure showing branching of the umbilical vessels into chorionic plate vessels. The stem villous vessels originate from chorionic plate vessels and form the villous tree (from Sastry B.V.R. Placental Pharmacology; New York; CRC Press; 1996, Chapter 3).

Effect of ADM on Normal and Preeclamptic Placental Arteries

Cumulative concentration-response curves were carried out using the thromboxane A_2 mimetic, U46619 ($1x10^{-9}$ to $1x10^{-5}$ mol/L) followed by a wash-out period of 60 minutes. The mean effective concentration that produced an 80% constriction (EC_{80}) was determined for each individual artery. Arteries were then pre-constricted with the EC_{80} dose of U46619 (7 minutes) and a cumulative ADM concentration-response curve was completed ($1x10^{-9}$ to $3x10^{-7}$ mol/L, 5-minute increments). A time-control experiment was performed on a parallel preparation of the same artery without added ADM (time control).

Statistical Analysis

A repeated measures analysis of variance was used to asses the relaxation response with ADM or without (time control) in both normal and preeclamptic placentas. A repeated measures analysis of variance was also used to evaluate the constriction response to U46619 in both normal and preeclamptic pregnancies. $P<0.05$ was considered significant.

Experiment C - Mechanism of ADM-Induced Relaxation in Placental Arteries

Effect of nitric oxide synthase inhibition on ADM-induced relaxation

Cumulative concentration-response curves were generated using U46619 ($1x10^{-9}$ to $1x10^{-5}$ mol/L) followed by a wash-out period of 60 minutes. The mean effective concentration that produced an 80% constriction (EC_{80}) was determined for each individual artery. Parallel preparations of the same artery were then incubated with N^G-monomethyl-L-arginine (L-NMMA) or N^G-monomethyl-D-arginine (D-NMMA) (10^{-4}

mol/L, 15 minutes). Arteries were then pre-constricted with the EC_{80} dose of U46619 (7 minutes) and cumulative ADM concentration-response curves were completed ($1x10^{-9}$ to $3x10^{-7}$ mol/L, 5-minute increments).

Effect of endothelial removal on ADM-induced relaxation

Parallel preparations of the artery were performed with either the endothelium intact or denuded. Removal of the endothelium was achieved by passing a human hair through the lumen of the artery. Successful removal of the endothelium was confirmed using immunohistochemistry (see below). Cumulative concentration-response curves to U46619 were produced as outlined above. Arteries were then pre-constricted with the EC_{80} dose of U46619 (7 minutes) and cumulative ADM concentration-response curves were completed.

Effect of inducible nitric oxide synthase inhibition on ADM-induced relaxation in endothelial denuded arteries

Parallel preparations of the artery were performed with the endothelium removed in both arteries. Cumulative concentration-response curves to U46619 were constructed as outlined above. Parallel preparations of the same artery were then incubated with the specific iNOS inhibitor (40) N-(3-(Aminomethyl)benzyl) acetamidine - dihydrochloride (1400W) (3×10^{-5} mol/L, 15 minutes) or HEPES-PSS buffer. Arteries were then pre-constricted with the EC_{80} dose of U46619 (7 minutes) and cumulative ADM concentration-response curves were completed.

Effect of nitric oxide synthase inhibition on ADM-induced relaxation in endothelial denuded arteries

Parallel preparations of the artery were performed with the endothelium removed in both arteries. Cumulative concentration-response curves to U46619 were constructed as outlined above. Parallel preparations of the same artery were then incubated with N^G-monomethyl-L-arginine (L-NMMA) or N^G-monomethyl-D-arginine (D-NMMA) (10^{-4} mol/L, 15 minutes). Arteries were then pre-constricted with the EC_{80} dose of U46619 (7 minutes) and cumulative ADM concentration-response curves were completed.

Effect of nitric oxide synthase inhibition and a non-selective endothelin receptor antagonist on ADM-induced relaxation in endothelial intact arteries

Cumulative concentration-response curves to U46619 were constructed as outlined above. Parallel preparations of the same artery were then incubated with N^G-monomethyl-L-arginine (L-NMMA) or N^G-monomethyl-D-arginine (D-NMMA) (10^{-4} mol/L, 15 minutes) plus PD 142893 ($3x10^{-6}$ mol/L, 15 minutes). PD 142893 is a non-selective endothelin receptor antagonist. Arteries were then pre-constricted with the EC_{80} dose of U46619 (7 minutes) and cumulative ADM concentration-response curves were completed.

Immunohistochemistry

A number of arteries were examined to determine if the endothelium had been effectively removed. Following the experiment the arteries were transferred from the wire myograph into tissue-freezing medium and kept frozen at -80°C until needed. The specimens were

cut into 12 μm sections, mounted on glass slides at -20°C, and stored at -80°C prior to staining. The presence of endothelial cells was verified visually by staining for the presence of von Willebrand's Factor (1:200; Beckman Coulter, Missisauga, Ont, Canada). Immunostaining was done using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). Slides were counterstained using an equal mixture of alcian blue and methyl green. The following control procedure was undertaken to ensure the specificity of the immunostaining. Control sections were subjected to identical staining procedures, except the primary antibody was replaced with either non-specific mouse IgG (1:1000) or blocking serum. No positive immunostaining was observed in either of these control slides. Comparisons were made between the difference in intensity of staining between paired arteries (intact or denuded).

Solutions for Experiments B and C

The arteries were bathed in HEPES-PSS containing (mmol/L) sodium chloride 142, potassium chloride 4.7, magnesium sulfate 1.17, calcium chloride 1.56, potassium phosphate 1.18, HEPES 10, and glucose 5.5. The potassium chloride-depolarizing solution was made using equimolar replacement of sodium chloride with potassium chloride.

Drugs for Experiments B and C

U46619 (Cayman Chemical Co, Ann Arbor MI., USA) stock solutions were prepared in methyl acetate. ADM (Phoenix Pharmaceuticals, Mountain View, CA., USA) was obtained in lyophilized aliquots in order to use fresh ADM for each experiment. L-

NMMA and D-NMMA (Calbiochem, LaJolla, CA., USA) stock solutions were prepared in distilled water. 1400W (Alexis Biochemicals Corporation, San Diego, CA., USA) stock solutions were prepared in distilled water. PD 142893 (Sigma, Oakville, ON., Canada) stock solutions were prepared in distilled water. Further dilutions were made with HEPES-PSS for all drugs.

Statistical analysis

A 2-way repeated measures ANOVA was used to compare the relaxation response in each of the parallel preparations. A Student-Newman-Keuls test was used to perform all pairwise multiple comparisons. $P<0.05$ was considered significant.

CHAPTER THREE

RESULTS

Experiment A

Subjects

Table 2 summarizes the characteristics of the patient groups. Comparisons were made between the PIH and preeclamptic group to the normotensive group. Maternal age, hematocrit, and hemoglobin were comparable between all groups. Parity was significantly lower in women with preeclampsia ($P<0.05$).

Maternal Plasma ADM Levels in Normotensive, PIH, and Preeclamptic Pregnancies

Comparison of plasma ADM levels in normotensive, PIH, and preeclamptic patients by gestational age grouping are shown in Figure 5. Plasma levels for gestational ages 25-30 weeks, 31-36 weeks, and 37-41 weeks in mol/L were: normotensive $4.6 \times 10^{-11} \pm 0.3$, $6.6 \times 10^{-11} \pm 0.7$, and $5.6 \times 10^{-11} \pm 0.2$; PIH $5.1 \times 10^{-11} \pm 0.7$, $6.1 \times 10^{-11} \pm 0.4$, and $6.0 \times 10^{-11} \pm 0.4$; preeclampsia $5.1 \times 10^{-11} \pm 0.3$, $6.3 \times 10^{-11} \pm 0.5$, and $5.9 \times 10^{-11} \pm 0.4$. There were no significant differences between groups. Values are expressed as mean ± standard error of mean.

Experiment B

Subjects

Table 3 summarizes the characteristics of women with preeclampsia and uncomplicated pregnancies. Maternal age, parity, gravidity, hematocrit, and hemoglobin were comparable between the 2 groups. Gestational age at delivery and infant birth weight were significantly lower in the preeclampsia group ($P<0.05$). Women with preeclampsia

Table 2. Characteristics of Patient Groups for Experiment A

Characteristic	Normal Pregnant (n=96)	Pregnancy Induced Hypertension (n=42)	Preeclamptic (n=37)
Maternal age, years	30.0 ± 0.5	31.5 ± 1.0	28.9 ± 0.9
Term blood pressure, mmHg	<140/90	145/96 ± 2/2	153/98 ± 2/2
Parity	1.0 ± 0.1	0.8 ± 0.1	0.5 ± 0.1*
Gravidity	2.6 ± 0.2	2.2 ± 0.2*	2.1 ± 0.2*
Proteinuria (≥+1 on urine testing)	(0/96)	(0/42)	(37/37)
Urate (umol/L)	ND	323.7±20.4(26/42)	334.0±14.5(27/37)
Hematocrit	0.36 ± 0.01(72/96)	0.37 ± 0.01(38/42)	0.36 ± 0.01(35/37)
Hemoglobin, g/L	122.3 ± 1.3(72/96)	123.2 ± 2.4(38/42)	120.8 ± 2.2(35/37)

Values are expressed as mean ± SEM. ND indicates not determined. (x/x) denominator denotes total number of patients, numerator denotes the number of patients with the available measurements.

*P<0.05 compared with normal pregnant.

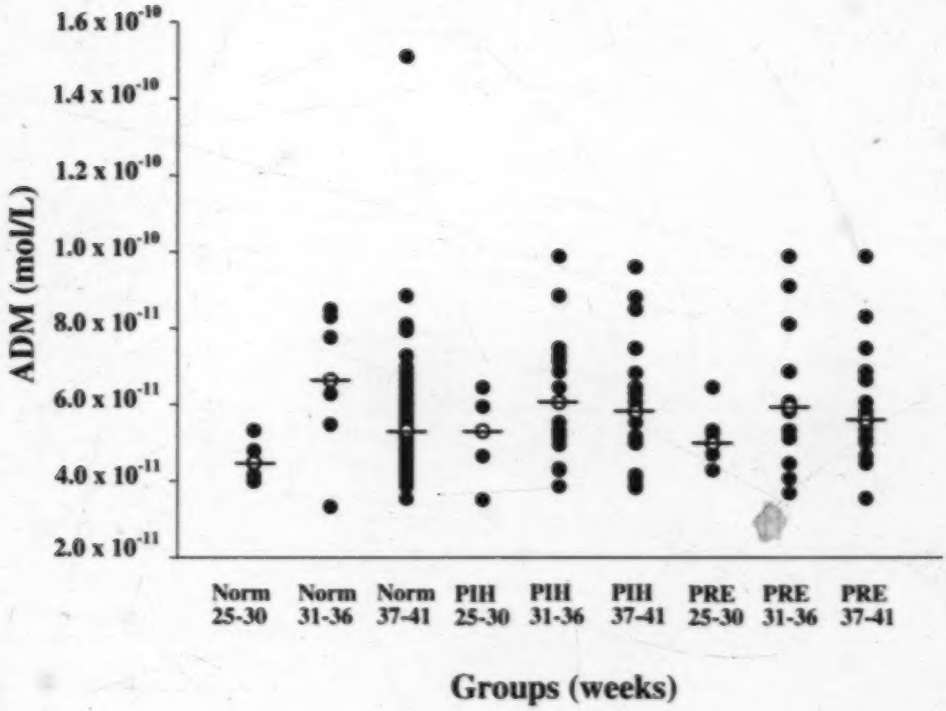

Figure 5. Maternal plasma adrenomedullin concentrations. Normotensive pregnancies (Norm) at 25-30 weeks (n=4); 31-36 weeks (n=7); 37-41 weeks (n=87). Pregnancy induced hypertension (PIH) at 25-30 weeks (n=4); 31-36 weeks (n=17); 37-41 weeks (n=21). Preeclamptic pregnancies (PRE) at 25-30 weeks (n=7); 31-36 weeks (n=14); 37-41 weeks (n=16). Open circles with horizontal bars: medians. Black circles: represent n numbers.

Table 3. Characteristics of Patient Groups for Experiment B

Characteristic	Normal Pregnant (n=12)	Preeclamptic (n=9)
Maternal age, years	26.3 ± 2.1	27.9 ± 1.6
Term blood pressure, mmHg	118/71 ± 2/2	166/111 ± 8/4*
Parity	0.8 ± 0.2	0.6 ± 0.4
Gravidity	2.7 ± 0.7	2.0 ± 0.4
Proteinuria (≥+1 on urine testing)	(0/12)	(9/9)
Urate (umol/L)	ND	316.1 ± 23.2 (7/9)
Hematocrit	0.37 ± 0.01 (6/12)	0.37 ± 0.02 (8/9)
Hemoglobin, g/L	124.7 ± 3.7 (6/12)	124.8 ± 5.8 (8/9)
Gestational age at delivery, wk	38.8 ± 0.4	36.2 ± 0.8 *
Infant birth weight, kg	3.4 ± 0.1	2.6 ± 0.3 *

Values are expressed as mean ± SEM. ND indicates not determined. (x/x) denominator denotes total number of patients, numerator denotes the number of patients with the available measurements.

*P<0.05 compared with normal pregnant.

had significantly higher systolic and diastolic blood pressure when compared to normotensive pregnant women ($P<0.05$).

Effect of U46619 and Potassium Chloride-Depolarizing Solution on Normal and Preeclamptic Placental Arteries

Concentration-dependent constriction occurred in the presence of U46619 ($1x10^{-9}$ to $1x10^{-6}$ mol/L). There was no significant difference in the EC_{80} value between normal ($4.04 \times 10^{-8} \pm 0.86$ mol/L) and preeclamptic arteries ($3.73 \times 10^{-8} \pm 0.37$ mol/L) (Figure 6). Maximum tension development was significantly greater in normal (2.74 ± 0.24 mN/mm) compared to preeclamptic arteries (1.80 ± 0.28 mN/mm; $P<0.05$) (Figure 7). In response to potassium chloride-depolarizing solution (140 mmol/L), maximum tension development was greater in normal (3.03 ± 0.26 mN/mm) compared to preeclamptic arteries (1.90 ± 0.36 mN/mm; $P<0.05$).

Effect of ADM on Normal and Preeclamptic Placental Arteries

Concentration-dependent relaxation occurred in the presence of ADM ($1x10^{-9}$ to $3x10^{-7}$ mol/L) when compared to its time-control in normal and preeclamptic placental arteries ($P<0.05$). There was a time-dependent effect of treatment on percent relaxation as indicated by the treatment and time interaction obtained in normal placental arteries. Similarly, in the preeclamptic group, the treatment and time interaction effect was also significant. Figure 8A illustrates the relaxation response between the time-control and ADM for normal placental arteries. Relaxation with the highest dose of ADM ($3x10^{-7}$ mol/L) was $42 \pm 6\%$ compared to its respective time-control $14 \pm 5\%$ of U46619-induced

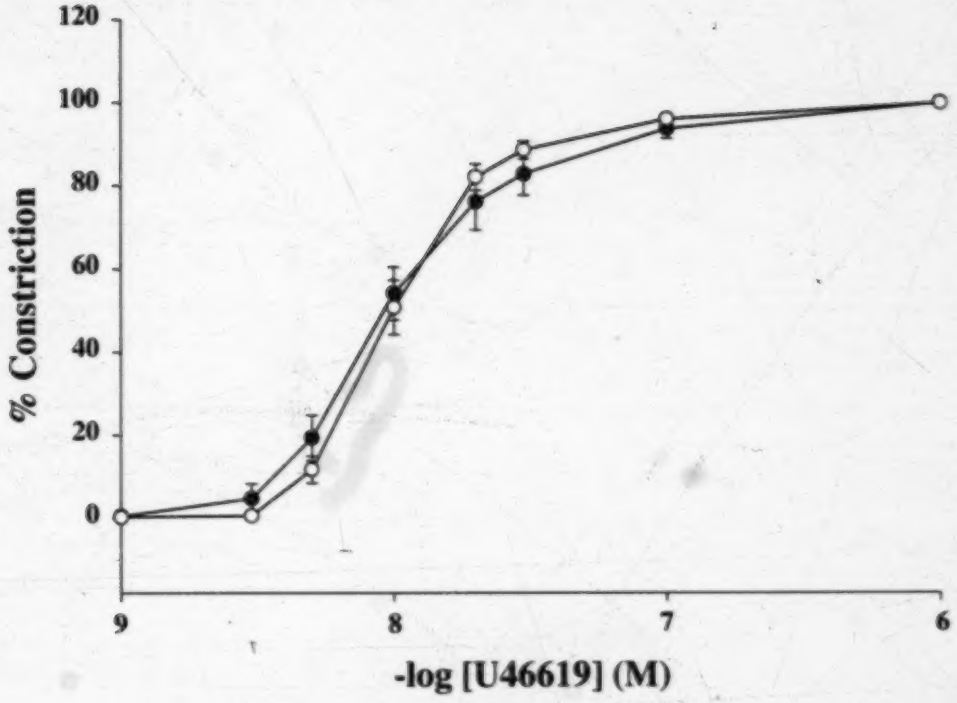

Figure 6. U46619-induced contraction in placental arteries from normotensive and preeclamptic pregnancies. Responses are expressed as a percentage of constriction. Black circles: normotensive group (n=12). Open circles: preeclamptic group (n=9). Vertical bars delineate standard error of mean.

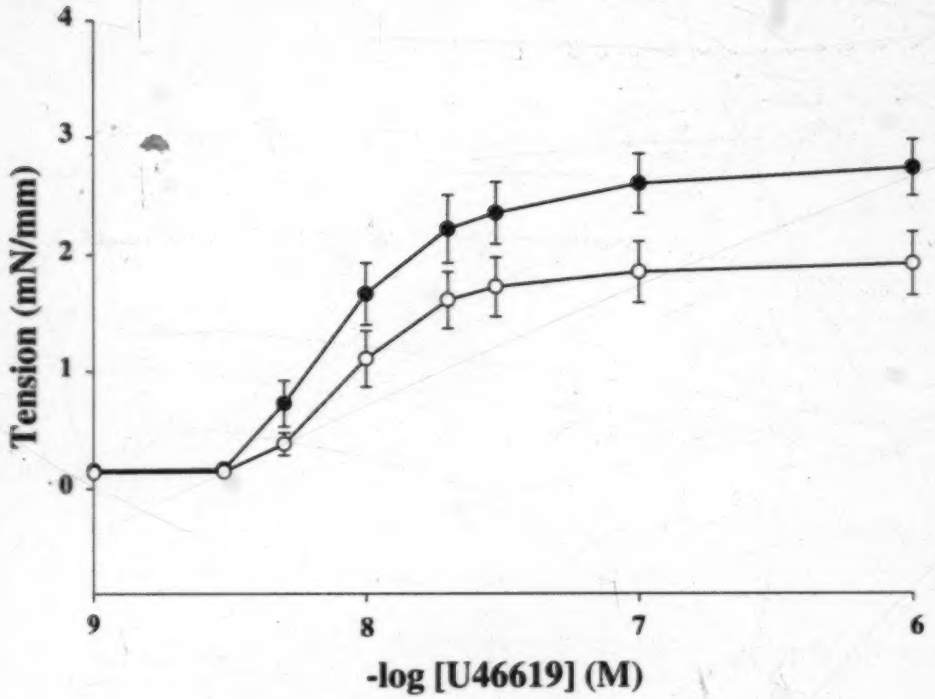

Figure 7. U46619-induced contraction in placental arteries from normotensive and preeclamptic pregnancies. Responses are expressed in absolute tension, mN/mm. Black circles: normotensive group (n=12). Open circles: preeclamptic group (n=9). Vertical bars delineate standard error of mean.

contraction. Figure 8B illustrates the relaxation response between the time-control and ADM for preeclamptic placental arteries. Relaxation at the highest dose of ADM ($3x10^{-7}$ mol/L) was 26 ± 11% compared to its respective time-control -1 ± 7% of U46619-induced contraction. The behavior of these two-way interaction effects in normal and preeclamptic groups was not statistically different as shown by the three-way interaction effect between treatment, time, and group. This was supported by a similarity in the dose response curves depicted in figure 8A and B.

Experiment C - Mechanism of ADM-Induced Relaxation in Placental Arteries

Subjects

Table 4 summarizes the characteristics of the normal pregnant women in each of the protocols.

Effect of nitric oxide synthase inhibition on ADM-induced relaxation

ADM-induced relaxation of U46619-preconstricted arteries was significantly inhibited by L-NMMA (8 ± 4%) compared to its control incubated with the inactive isomer D-NMMA (19 ± 4%), at the highest dose of ADM ($3x10^{-7}$ mol/L) (Figure 9).

Effect of endothelial removal on ADM-induced relaxation

There was no significant difference in relaxation response to ADM between endothelium-intact (25 ± 6%) and endothelial denuded (25 ± 4%) U46619-preconstricted arteries (Figure 10).

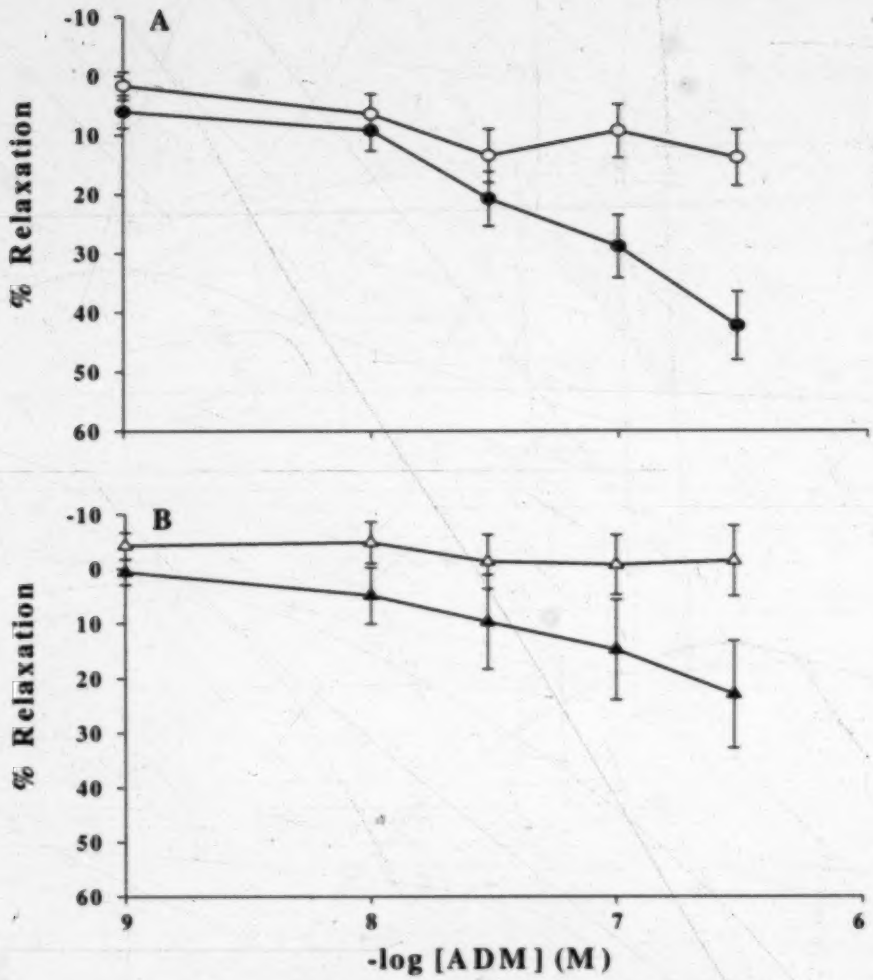

Figure 8. Effect of adrenomedullin on placental arteries from normotensive and preeclamptic pregnancies. **Panel A**, Open circles: *normotensive* time-control group (n=12). Black circles: *normotensive* ADM group (n=12). **Panel B**, Open triangles: *preeclamptic* time-control group (n=9). Black triangles: *preeclamptic* ADM group (n=9). Vertical bars delineate standard error of mean. Responses are expressed as a percentage of relaxation from U46619-preconsticted levels.

Table 4. Characteristics of Patient Groups for Experiment C

Characteristic	L-NMMA (n=14)	Endothelium Intact or Denuded (n=12)	1400W (n=11)	L-NMMA in Denuded (n=7)	L-NMMA + PD 142893 (n=9)
Maternal age, years	26.9 ± 1.4	33.0 ± 1.7	27.7 ± 1.8	30.7 ± 2.0	26.4 ± 2.2
Term blood pressure, mmHg	114/72 ± 2/2	121/74 ± 2/2	117/76 ± 4/3	111/75 ± 4/2	112/72 ± 4/4 (7/9)
Parity	1.0 ± 0.3	1.3 ± 0.4	1.3 ± 0.3	0.6 ± 0.4	0.7 ± 0.2
Gravidity	2.6 ± 0.4	3.3 ± 0.4	2.8 ± 0.3	2.3 ± 0.8	2.4 ± 0.5
Gestational age at delivery, wk	37.7 ± 0.4	39.3 ± 0.3	38.9 ± 0.3	38.9 ± 0.3	39.0 ± 0.4
Infant birth weight, kg	3.4 ± 0.1	3.6 ± 0.1	3.7 ± 0.1	3.5 ± 0.1	3.3 ± 0.1
Hematocrit	0.36 ± 0.01 (7/14)	0.38 ± 0.00 (8/12)	0.34 ± 0.01 (7/11)	0.36 ± 0.04 (2/7)	0.36 ± 0.01 (4/9)
Hemoglobin, g/L	121.0 ± 2.2 (7/14)	125.8 ± 5.4 (10/12)	118.5 ± 5.6 (8/11)	117.0 ± 10.0 (2/7)	122.5 ± 5.1 (2/7)

Values are expressed as mean ± SEM. (x/x) denominator denotes total number of patients, numerator denotes the number of patients with the available measurements.

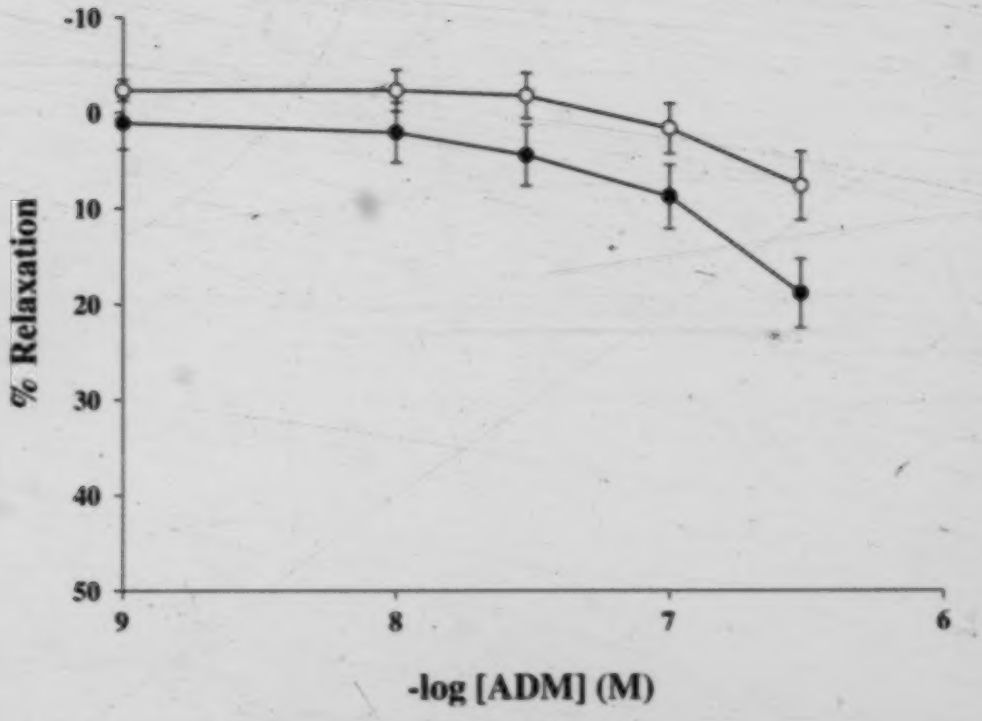

Figure 9. Effect of nitric oxide inhibition on adrenomedullin-induced relaxation in normal placental arteries. Open circles: arteries incubated with L-NMMA (n=14). Black circles: arteries incubated with D-NMMA (n=14). Vertical bars delineate standard error of mean. Responses are expressed as a percentage of relaxation from U46619-preconstricted levels.

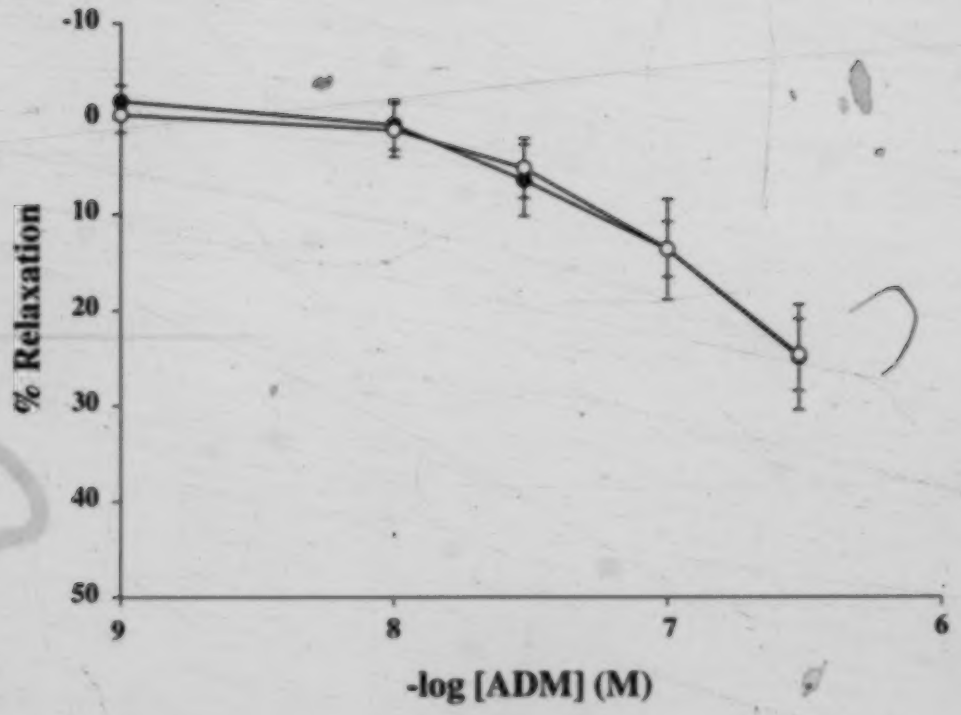

Figure 10. Effect of endothelial removal on adrenomedullin-induced relaxation in normal placental arteries. Open circles: endothelial denuded arteries (n=12). Black circles: endothelium-intact arteries (n=12). Vertical bars delineate standard error of mean. Responses are expressed as a percentage of relaxation from U46619-preconstricted levels.

Effect of inducible nitric oxide synthase inhibition on ADM-induced relaxation in endothelial denuded arteries

There was no significant difference in relaxation to ADM in U46619-preconstricted arteries treated with the iNOS inhibitor, 1400W (18 ± 7%) compared to ADM-induced relaxation in the absence of iNOS inhibition (24 ± 4%) (Figure 11).

Effect of nitric oxide synthase inhibition on ADM-induced relaxation in endothelial denuded placental arteries

There was no significant difference in relaxation to ADM in U46619-preconstricted arteries treated with L-NMMA (23 ± 7%) compared to D-NMMA (17 ± 7%) (Figure 12).

Effect of nitric oxide synthase inhibition and the non-selective endothelin receptor antagonist, PD 142893, on adrenomedullin-induced relaxation in endothelial intact arteries

Relaxation of arteries was significantly inhibited by L-NMMA plus PD 142893 (7 ± 3%) compared to its control incubated with the inactive isomer D-NMMA plus PD 142893 (21 ± 4%) at the highest dose of ADM ($3x10^{-7}$ mol/L) (Figure 13A). There was no significant difference in relaxation between the arteries treated with L-NMMA/D-NMMA plus PD 142893 (Figure 13A) compared to endothelium-intact arteries incubated with L-NMMA/D-NMMA alone (Figure 13B).

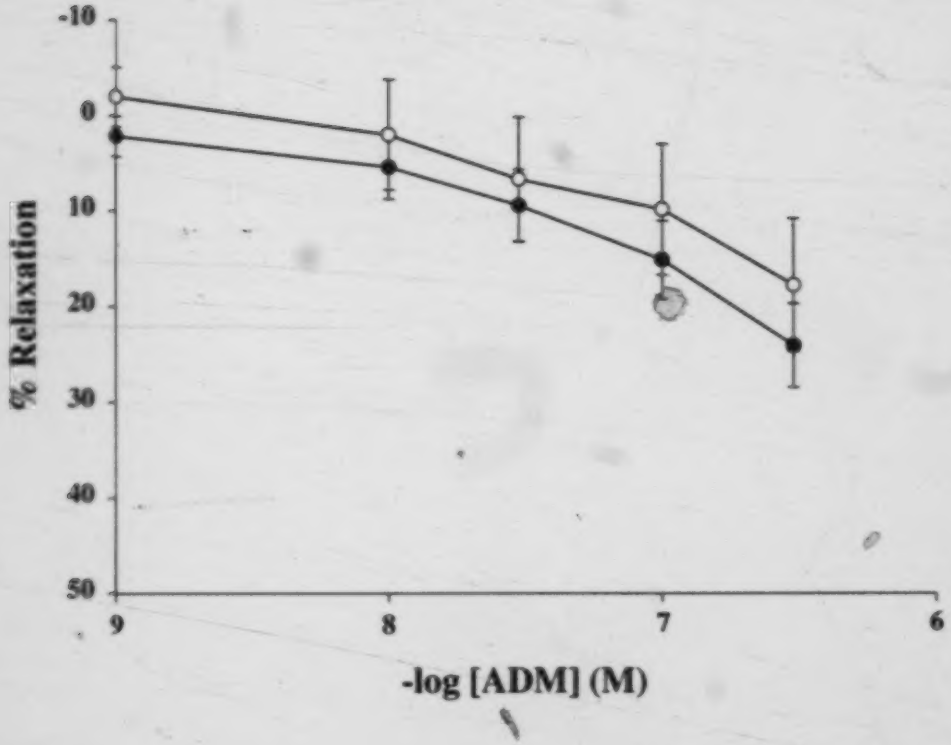

Figure 11. Effect of inducible nitric oxide inhibition on adrenomedullin-induced relaxation in endothelial denuded normal placental arteries. Open circles: arteries incubated with 1400W (n=11). Black circles: arteries incubated with HEPES-PSS (n=11). Vertical bars delineate standard error of mean. Responses are expressed as a percentage of relaxation from U46619-preconstricted levels.

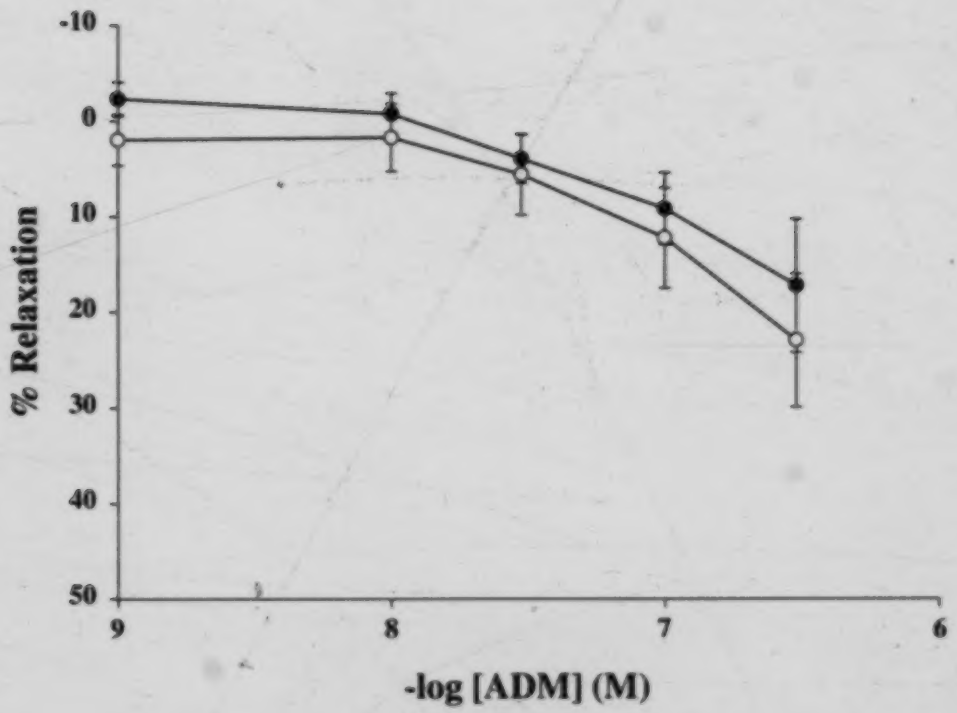

Figure 12. Effect of nitric oxide inhibition on adrenomedullin-induced relaxation in endothelial denuded normal placental arteries. Open circles: arteries incubated with L-NMMA (n=7). Black circles: arteries incubated with D-NMMA (n=7). Vertical bars delineate standard error of mean. Responses are expressed as a percentage of relaxation from U46619-preconstricted levels.

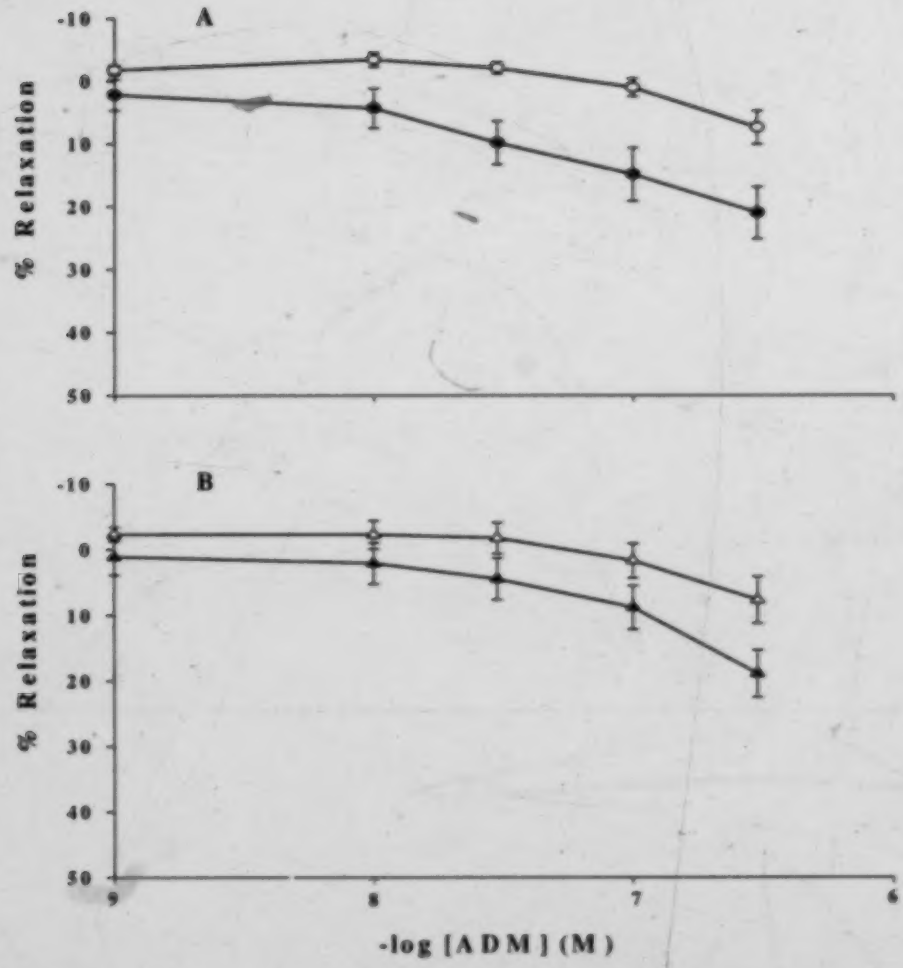

Figure 13. Effect of nitric oxide inhibition and non-selective endothelin receptor antagonist on adrenomedullin-induced relaxation in endothelial intact placental arteries. **Panel A,** Open circles: arteries incubated with L-NMMA plus PD 142893 (n=11). Black circles: arteries incubated with D-NMMA plus PD 142893 (n=11). **Panel B,** Open triangles: arteries incubated with L-NMMA (n=14). Black triangles: arteries incubated with D-NMMA (n=14). Vertical bars delineate standard error of mean. Responses are expressed as a percentage of relaxation from U46619-preconstricted levels.

Immunohistochemistry

Immunohistochemistry revealed a substantial reduction in staining for von Willebrand's Factor in denuded arteries compared to intact arteries. This reduction in staining is illustrated in figure 14A and B. A representative sample of arteries was analyzed by immunohistochemistry for endothelial removal (4 out of 12 intact arteries and 6 out of 12 denuded arteries were examined).

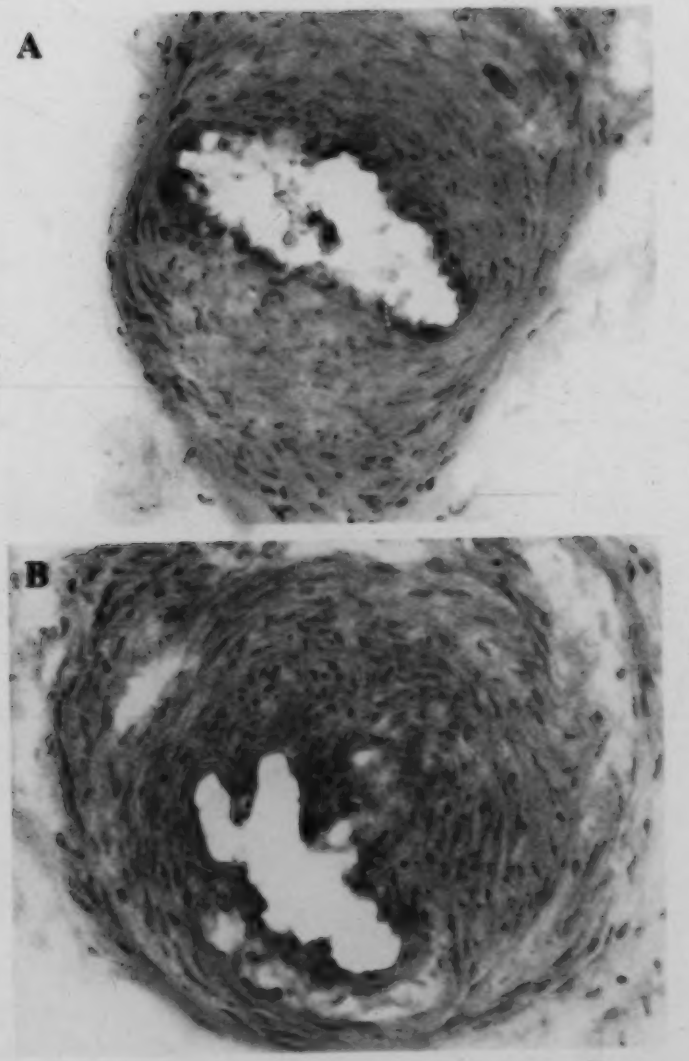

Figure 14. Immunostaining for von Willebrand's Factor to confirm removal of the endothelium. Representative sections of stem villous arteries from normotensive pregnant women at 400X magnification **(A)** endothelium intact **(B)** endothelium denuded.

CHAPTER FOUR

DISCUSSION

AND

CONCLUSIONS

This study supports those previous findings of unchanged maternal plasma levels of ADM in normotensive and preeclamptic pregnancies (26, 104). In addition, we have found no difference in maternal plasma levels of ADM in the PIH group. The PIH subgroup was included due to the potential difference in the pathophysiological mechanism underlying this condition as compared to that of preeclampsia (20, 154) and had not previously been studied.

Other clinical conditions of hypertension including essential hypertension (60, 77, 161), renal failure (60), heart failure (122), and primary aldosteronism (69) all show an increase in plasma ADM levels. In these hypertensive conditions ADM may be involved in a compensatory mechanism against the elevation in blood pressure. We have demonstrated that in preeclampsia and PIH, there is no compensatory increase in plasma ADM. Consequently, this absence of a compensatory effect could potentially contribute to the hypertension seen in PIH and preeclampsia.

Since the placenta is devoid of any autonomic innervation (137) locally produced vasoactive factors are essential in maintaining the low vascular resistance characteristic of the placental circulation. A paracrine or autocrine mode of action of ADM has been inferred from the elevated concentrations of this peptide found in fetoplacental tissues (98). We have shown that ADM induces relaxation in placental arteries from both normotensive and preeclamptic pregnancies. We originally formulated the hypothesis that an attenuated response of ADM-induced relaxation would exist in placental arteries from preeclamptic pregnancies compared to normotensive pregnancies. However, we found no

significant difference in ADM-induced relaxation between these groups. It has been previously reported that ADM levels are increased in amniotic fluid and umbilical vein plasma in women with preeclampsia compared to normotensive pregnancy, suggesting that greater concentrations of ADM are available locally in preeclampsia (26). ADM expression is also increased in fetoplacental tissue from women with PIH (96). Considering placental arteries from preeclamptic pregnancies retain their ability to respond to ADM this could potentially reflect a compensatory mechanism to counteract those factors responsible for increasing vascular resistance in preeclampsia.

Although we did not find a difference in ADM-induced relaxation between normotensive and preeclamptic groups, interestingly we did find that in response to the TxA_2 mimetic U46619, and to the potassium chloride-depolarizing solution, the tension developed in placental arteries from preeclamptic pregnancies was significantly less compared with arteries from normotensive pregnancies. Therefore, altered vasoconstrictor activity may exist in response to vasoactive agents in placentas obtained from women with preeclampsia. Similar to our results, a placental lobule perfusion method demonstrated a reduced pressure increase created by U46619 in placentas from women with preeclampsia than from normotensive pregnancies (135). In addition, the constrictor response elicited in umbilical arteries from women with preeclampsia demonstrated a decreased sensitivity to a potassium chloride-depolarizing solution (8, 46). The decreased constrictor response to these agents in preeclampsia suggests a compensatory mechanism to counteract the increased resistance seen in this condition. However, it has been reported that there is no difference in vasoreactivity to various constrictor and dilator agents in chorionic plate

arteries from normal or preeclamptic pregnancies (57). Therefore, altered vasoreactivity exists in response only to certain agents and perhaps only in particular placental vessels.

A further objective of this study was to investigate the mechanism of ADM-induced relaxation in placental arteries from normotensive pregnancies. Figure 15 illustrates the hypotheses considered from our studies. NO has been shown to be a significant factor in maintaining the low vascular resistance within the placenta (73, 112, 133). The majority of NOS activity in the placenta is calcium-dependent and therefore would correlate with the eNOS isoform (114). In support of this, eNOS has been identified and purified, in the human placenta, with no indication of the presence of other NOS isoforms (39). On the other hand, there is evidence for calcium-independent NOS activity (correlating to the iNOS isoform) within the villous vasculature (114, 156, 157). Nevertheless, eNOS has been localized in the endothelial cells of the resistance vasculature in the placenta and in the syncytiotrophoblast (113).

To examine the possible involvement of the NO pathway in ADM-induced relaxation we used the non-specific NOS blocker, L-NMMA. Experimental evidence has shown that the contribution of NO to ADM-induced relaxation is dependent on the vascular bed and the species being studied (128, 160). We found that within human placental arteries ADM-induced relaxation was attenuated after pre-incubation with L-NMMA. These findings demonstrate that ADM-induced relaxation within human placental arteries is, at least in part, mediated by NO.

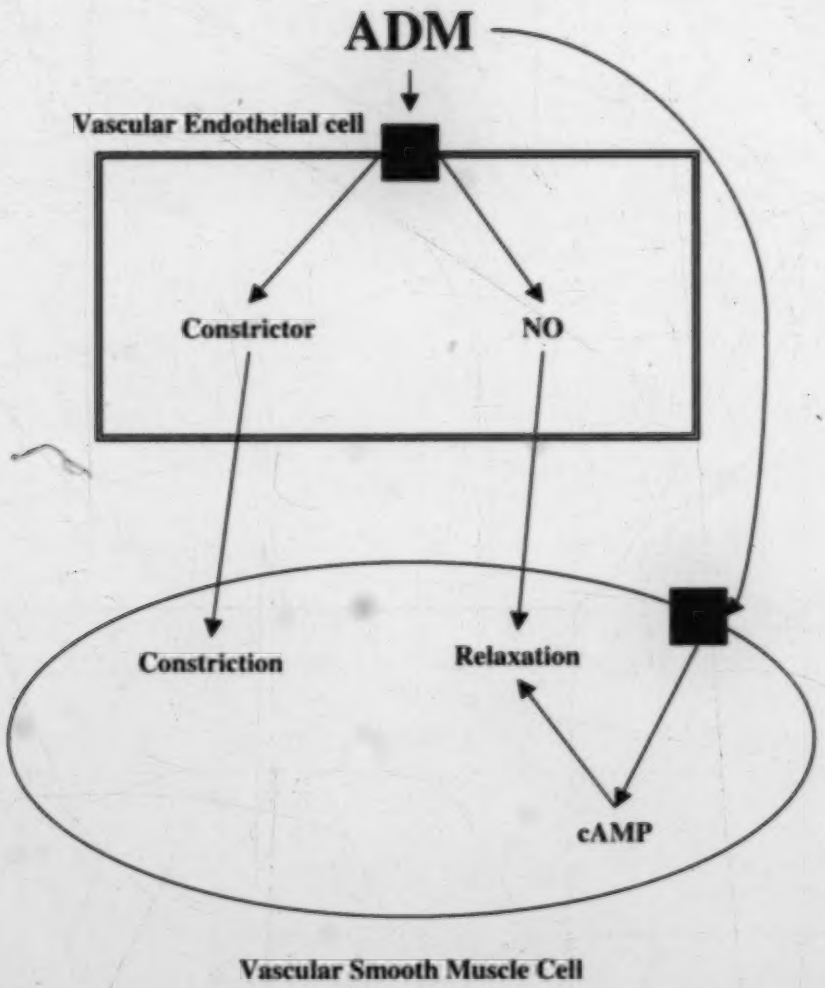

Figure 15. Hypothesized mechanisms of adrenomedullin-induced relaxation in placental arteries.

The endothelium is not only important for maintaining vessel wall integrity, but can influence vascular tone by releasing vasoactive agents, such as NO (14). Our next step was to examine the importance of the endothelium in ADM-induced relaxation. In rat aortic rings, canine mesenteric arteries, and canine femoral veins, removal of the endothelium attenuates ADM-induced vasorelaxation (6, 48), whereas in canine femoral arteries ADM-induced relaxation was independent of the endothelium (6). We found, in human placental arteries, that there was no significant difference in ADM-induced relaxation between intact and denuded arteries, suggesting that the endothelium does not contribute to ADM-induced relaxation. Since L-NMMA had attenuated the relaxant response to ADM, we postulated that the NO contribution to ADM-induced relaxation was derived from the VSMC of the vessel wall (Figure 15). Although, endothelial staining for von Willebrand's Factor has shown a significant decrease in staining in endothelial-denuded arteries compared to endothelium-intact arteries (Figure 14), only a few endothelial cells are required to induce a relaxation response. Therefore, interpretation of these results must be made with caution.

ADM-induced relaxation partially occurs via activation of adenylate cyclase and subsequent production of cAMP (32). cAMP has been shown to increase iNOS in unstimulated and stimulated VSMC (31, 56). It should be noted that cAMP can also cause relaxation independent of NOS. We postulated that ADM binds to its receptor on the VSMC and causes an increase in cAMP. The cAMP-induced iNOS production then subsequently causes relaxation. Our results showed that after removal of the endothelium plus incubation with a specific inhibitor of iNOS, 1400W (40), there was no significant

difference in relaxation to ADM between arteries with iNOS inhibition and those without. Therefore, we investigated the possible role of eNOS production of NO in the VSMC as the source of the vasodilator agent. After removal of the endothelium plus incubation with a non-specific inhibitor of NOS, L-NMMA, there was no significant difference in relaxation between arteries with L-NMMA and its respective control incubated with D-NMMA. The results suggest that neither eNOS nor iNOS contribute to ADM-induced NO production in the VSMC.

Although initially it appeared that ADM-induced relaxation in the placental arteries was independent of the endothelium, the endothelium itself produces other factors, including vasoconstrictive agents that can alter the tone of resistance vessels. We hypothesized that the endothelium releases a vasoconstrictive factor, notably ET, upon stimulation by ADM. ET is a potent vasoconstrictor agent released mainly from the endothelial cells. Although, ADM has been shown to inhibit ET release from VSMC (78), the effect of ADM on ET release from endothelial cells is not known. In addition, ET has been found to be one of the most potent vasoconstrictors studied in resistance arteries in the placental circulation (99), causing constriction by both receptor subtypes, ET-A and ET-B (150). The non-selective endothelin receptor antagonist, PD 142893, plus L-NMMA or D-NMMA resulted in no change in relaxation compared with L-NMMA or D-NMMA alone in endothelial-intact arteries. The results suggest that ADM-induced relaxation does not stimulate the release of ET from the endothelial cells. However, the possible release of other vasoconstrictive agents from the endothelium after stimulation by ADM requires further investigation.

We suggest that ADM-induced vasorelaxation occurs by direct stimulation of the release of cAMP in VSMC and by NO release from the endothelial cells, both of which initiate intracellular mechanisms causing relaxation. In addition, the release of an, as yet unknown vasoconstrictive factor from the endothelial cells, may partially counteract the relaxation caused by NO and/or cAMP. Therefore, removal of the endothelium eliminates all endothelium-derived vasoactive agents, both dilators and constrictors, the net effect of which is no change in the response to ADM. It is also possible that removal of the endothelium makes the ADM receptor on the VSMC, more accessible to ADM and therefore increases the contribution of the cAMP pathway to relaxation. This in turn compensates for the lack of NO from the endothelium.

In summary, although ADM levels are elevated in many other hypertensive disorders, we did not find a significant difference in maternal plasma concentrations in either preeclampsia or PIH compared with normal pregnancy. This lack of a compensatory response to the increased blood pressure that exists in these pathophysiological conditions may potentially contribute to the cardiovascular state of such patients. We have also shown, for the first time, that ADM causes a dose-dependent relaxation of placental arteries. We conclude that ADM-induced vasodilation may contribute to the low vascular resistance seen in normal pregnancy. Furthermore, retention of this vasorelaxant activity in placental arteries derived from preeclamptic pregnancies, may serve to attenuate the increase in placental vascular resistance associated with this condition. The mechanism of ADM-induced relaxation in placental arteries from normotensive pregnancies is partially dependent on NO release, most likely from the endothelial cells and cAMP from

receptors on VSMC. In addition, ADM may stimulate the release of a vasoconstrictive factor, that is yet unknown, which opposes relaxation.

Further studies may concentrate on clarification of the mechanism of ADM-induced relaxation and the identity of the vasoconstrictor released in response to ADM or greater access to ADM receptors. In the current studies, the focus was directed towards the NO contribution to ADM-induced relaxation. The NO pathway has been implicated in control of vascular resistance in many vascular beds and specifically has been found to have a role in maintaining the low vascular resistance characteristic of the placental circulation (112). Several studies have also focused on the role of NO in preeclampsia. However, it would also be of interest to investigate other possible mechanisms of ADM-induced relaxation, including the cAMP pathway. Since it is reported that levels of ADM are increased in other hypertensive disorders, it would be of interest to determine the levels of ADM in women with hypertension prior to pregnancy who remain hypertensive during pregnancy. We would expect ADM levels to be increased in such circumstances in comparison to the PIH and preeclamptic groups we studied.

ADVANTAGES AND DISADVANTAGES OF THE WIRE MYOGRAPH TECHNIQUE

When studying the effect of vasoactive substances on the placental circulation there are several methods that have been utilized. Placental or cotyledon perfusions study the function of the resistance vasculature with changes in perfusion pressure as the final outcome. The disadvantage of the whole cotyledon perfusion method is achieving perfusion to all vessels in the preparation. Under such conditions the intravascular volume of the placenta is unknown and the vasoactive substances may not be equally distributed within the preparation (134).

Organ bath studies have typically used umbilical artery rings. The umbilical artery is a conduit vessel and is not a major contributor to vascular resistance seen in the placenta. Therefore, techniques have been developed to study small resistance artery preparations. The ring-mounted preparation, or wire myograph technique (109) and the cannulated, or pressurized technique, are the two methods most commonly employed in *in vitro* investigations of resistance vessel vasoreactivtiy. The wire myograph technique enables measurement of isometric tension in small resistance vessels (109), and attempts to mimic the vessel tension that is seen in *in vivo* conditions. Disadvantages arising from this technique relate to the damage that is done to the endothelium and VSMC due to the wire cannulation through the vessel (34, 45). Investigators have reported slight distortion of mounted vessels at the wire contact points (99) plus a disruption to the natural shape of vessels, because the wires stretch the vessels in an oval shape. Although this method

attempts to mimic *in vivo* conditions, it is lacking in a critical stimulus for the release of vasoactive agents from the endothelium, namely blood flow. As previously mentioned, blood flow through the lumen of vessels creates endothelial shear stress, which is a known stimulant of NO release and possibly of ADM release (13, 84). Therefore, a major disadvantage of the wire myograph technique is the absence of flow-mediated shear stress.

The pressurized myograph preparation is a sophisticated method of evaluating vascular function in resistance vessels and is considered a more physiological method in comparison to the wire myograph technique. This system has the advantage of producing minimal damage to the endothelium, as nothing is passed completely through the lumen of the blood vessel. The pressurized system enables control of pressure and volume-flow within a cannulated vessel. Therefore, the pressurized system allows for more reliable responses to endothelium-dependent factors. The vessels also maintain their physiological shape and are not subjected to the abnormal vessel stretching that occurs in the wire myograph system (34). As this preparation enables the control of fluid flow through the lumen of the blood vessel, it also has the major advantage of permitting the effect of flow on the release of vasoactive agents to be controlled and assessed (45). The different strengths and weaknesses of the wire and pressure myograph system have to be weighed up in terms of the vessels and vasoactive agents being investigated and the known limitations of each technique. For example, responses to vasoactive agents can differ between wire and pressurized systems. Pressurized arteries from the rat are significantly more sensitive to norepinephrine compared to arteries mounted on a wire myograph

system, and these arteries also produce a contractile response to angiotensin II, whereas the arteries mounted on a wire myograph do not (34).

It is apparent that the technique chosen may have an effect on the results obtained, and therefore interpretations must be made with caution. The wire myograph technique was chosen for the current studies because the anatomy of the placental circulation is such that there is extensive branching of the resistance arteries, thus making use of these vessels unsuitable in a pressurized system since this system demands that the vessels have no leaks (Poston, L. personal communication).

CRITICAL REVIEW

The concentrations of ADM, used in the dose response curves, were higher than was observed in the plasma. ADM is a local or paracrine hormone and therefore acts at a local level. The concentrations measured in plasma may simply be the spillover from local production. Therefore, concentrations at the local level where ADM is functioning are, most likely, higher than those measured in the plasma.

In our studies, the placental arteries were pre-constricted with U46619. It is possible that in the presence of other vasoconstrictor agents the relaxation response to ADM may be different. Therefore, our conclusions are limited to arteries pre-constricted with U46619. In addition, basal perfusion pressure within the placenta does not appear to rely on eicosanoid synthesis (73, 99). Therefore the choice of pre-constricting the arteries with U46619 might be questioned, although, U46619 has been used extensively as a pre-constrictor when studying placental vessels (43, 55, 99).

It has been shown in placental artery preparations that inhibition of NO slightly increases basal tension (99). Therefore, interpretation of ADM-induced relaxation in the presence of L-NMMA must be made with caution. It is possible that L-NMMA may alter pre-constrictor tone of these arteries.

Our results indicate that removal of the endothelium results in no significant difference in ADM-induced relaxation. ADM may be able to bind to its receptor on the VSMC more

readily in endothelial-denuded arteries, considering the vascular smooth muscle was more exposed than in arteries where the endothelium was intact. Subsequently, this would result in increased production of cAMP in endothelial-denuded arteries compared to the intact arteries. This would give a false impression that there was no difference in relaxation between the two groups. Experiments are underway to determine the amount of cAMP generated from endothelium-intact versus denuded arteries stimulated by ADM.

Placental arteries do not respond consistently to endothelium-dependent vasodilators, such as acetylcholine (1, 11, 99, 143). Our own preliminary data suggest that placental arteries are not responsive to acetylcholine. Considering the importance of the endothelium and its involvement in releasing vasoactive agents, it is important to determine if the endothelium was intact and functional. Likewise, when the endothelium was denuded it was necessary to verify that it was removed successfully. Staining of the endothelium using a marker that is present only in the endothelium has been a method of choice for this verification process in placental vessels (99). Endothelial staining for von Willebrand's Factor has shown a significant decrease in staining in endothelial-denuded arteries compared to endothelium-intact arteries. However, caution must be taken with this method given that only a few endothelial cells are actually required to elicit an endothelial-dependent response. Therefore, we have also shown pharmacologically that the endothelium was removed from the arteries. In experiments using L-NMMA and D-NMMA with the endothelium removed, there was no significant difference in relaxation, whereas the use of endothelium-intact arteries showed a significant attenuation of the relaxation response with L-NMMA.

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APPENDIX

INTERNAL CIRCUMFERENCE: TENSION CHARACTERISTICS

Preliminary experiments were initially completed to confirm the optimal setting for placental arteries. Tension developed is due to both active and passive properties of the vessel wall. The passive tension is due to the elastic properties of the vessel wall, whereas active tension is due to the activity of the contractile properties of the VSMC (87). The active tension that is generated by the VSMC is directly related to the degree of overlap between actin and myosin filaments (63). When the filaments are too far apart, there is no overlap of the actin and myosin filaments, resulting in no cross-bridge formation. When the filaments are at an optimal length, the number of cross-bridge associations is increased and maximum tension can be generated. However, when the muscle filaments are compressed (or too close together) the actin filaments interfere with one another. This causes the tension developed to be less than maximal (132), because the cross-bridging is not optimal.

PROTOCOL FOR DETERMINATION OF OPTIMAL SETTING FOR PLACENTAL ARTERIES

- Mount artery and set at zero tension (wires touching)
- Warm up artery for 30 minutes in HEPES-PSS buffer, changing HEPES-PSS every 10 minutes

TOTAL CURVE:

- Replace HEPES-PSS with potassium chloride-depolarizing solution (140 mmol/L)

- Adjust micrometer in step-wise increments one notch (25μm) at a time (~9-10 notches in total) and record the diameter and tension at each point
- Reset artery back to starting point
- Remove potassium chloride-depolarizing solution and replace with HEPES-PSS
- 30 minute wash-out period (change HEPES-PSS every 10 minutes)

PASSIVE CURVE:

- Replace HEPES-PSS with calcium-free buffer with papaverine (10^{-4} mol/L)
- Incubate artery for 20 minutes (do not change buffer)
- Adjust micrometer in step-wise increments and record the diameter and tension at each point (same number of adjustments as the total curve)

Calculations: (example shown on Table 5 and Figure 16)

- Prepare graph (Figure 16):

  Total curve

  Passive curve

  Active curve = Total - Passive

- From graph chose internal circumference that produced the least passive tension and the most active tension (250 μm on Figure 16)
- Input paired data set of diameter and tension from passive curve into computer program (Table 5)
- Computer program uses the Law of LaPlace to calculate the point on the passive curve that corresponds to an effective transmural pressure of 40mmHg (placental circulation) (**Length @ 40mmHg**)
- This value is called the L40

- Program also gives you the length at each point of the passive curve (**Length** column)
- In the **Displacement** column the diameter that was chosen (250 µm), to produce the most active tension and the least active tension, is divided by the **Length @ 40mmHg**
- This value will be the percent of the L40 that is required to give you an optimal setting: 628.54/658.54 x 100 = 95%
- In this example the optimal setting is 95% of L40
- Our preliminary experiments resulted in an optimal setting of 96 ± 4% of L40 (n=5). We choose to use 90% of L40 considering it is published in scientific papers (99).

Table 5. Internal Circumference: Tension Characteristics - Computer Program

Displacement	Force	Length	Tension	LaPlaceT@40mmHg
175	0.052	478.54	0.1288821	0.4060948
200	0.064	525.54	0.1586242	0.4485255
225	0.100	578.54	0.2478503	0.4909561
250	0.148	**628.54**	0.3668184	0.5333866
275	0.260	678.54	0.6444108	0.5758173
300	0.444	728.54	1.100455	0.6182479
325	0.764	778.54	1.893576	0.6606785

Length @ 40 mmHg = 658.54

Displacement @ 40 mmHg = 265

.9L = 592.686

Displacement @ .9L = 232.073

Force @ .9L = .123198

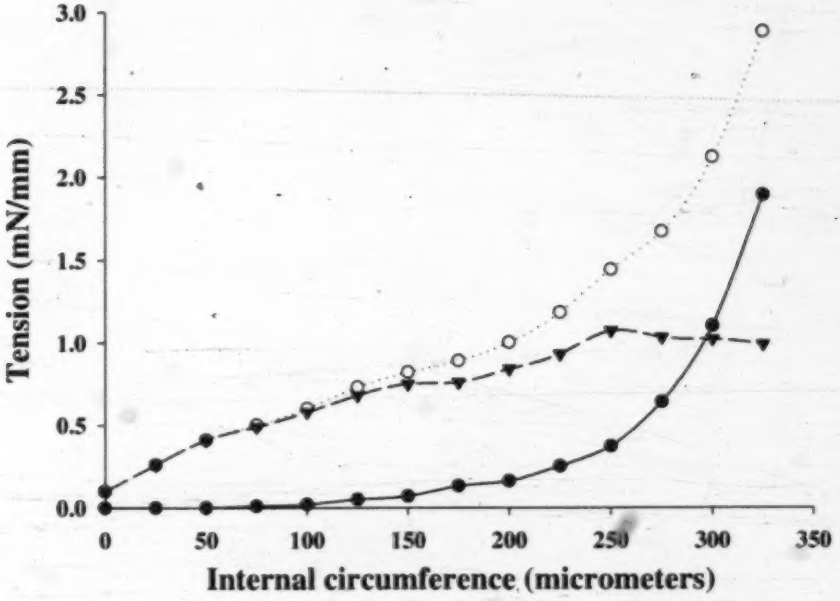

Figure 16. Internal circumference tension characteristics of a placental artery. Open circles: total curve. Black circles: passive curve. Black triangles: active curve (total - passive).